B4301

REVERSAL OF EFFLUX PUMP-MEDIATED MULTIDRUG RESISTANCE IN BACTERIA AND TUMOUR CELLS

Ph.D. Thesis

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Szeged

2006

B4301

I dedicate this thesis to the memory of my mother.

List of publications incorporated in the thesis

Kawase M, Motohashi N, Sakagami H, Kanamoto T, Nakashima H, Ferenczy L, **Wolfart K**, Miskolci C, Molnar J. Antimicrobial activity of trifluoro-methylketones and their synergism with promethazine. *Int J Antimicrob Agents*. 2001 18(2): 161-5

Misbahi H, Brouant P, Hever A, Molnar A, **Wolfart K**, Spengler G, Mefatah H, Molnar J, Barbe J. Benzo[b]- 1,8- naphthyridine derivatives: synthesis and reversal activity of multidrug resistance. *Anticancer Res.* 2002 22(4): 2097-101

Gallo S, Atifi S, Mahamoud A, Santelli-Rouvier C, **Wolfart K**, Molnar J, Barbe J. Synthesis of aza mono, bi and tricyclic compounds. Evaluation of their anti MDR activity. *Eur J Med Chem.* 2003 38(1): 19-26

Wolfart K, Molnar A, kawase M, Motohashi N, Molnar J. Effects of trifluoro-methylketones on the motility of *Proteus vulgaris*. Biol Pharm Bull. 2004 27(9): 1462-4

List of publications related to the thesis

Shah A, Gavieriya h, Motohashi N, Kawase M, Saito S, Sakagami H, Satoh K, Tada Y, Solymosi A, **Wolfart K**, Molnar J. 3,5-diacetil-1,4-dihydropiridines: synthesis and MDR reversal in tumor cells. *Anticancer Res.* 2000 20: 373-8

Motohashi N, Kawase M, Shirataki Y, Tani S, Saito S, Sakagami H, Kurihara T, Nakashima H, **Wolfart K**, Mucsi I, Varga A, Molnar J. Biological activity of feijoa peel extracts. *Anticancer Res.* 2000 20(6B): 4323-9

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Shirataki Y, Motohashi N, Tani S, Sunaga K, Sakagami H, Satoh K, Nakashima H, Kanamoto T, **Wolfart K**, Molnar J. Antioxidative activity of *Allium victorialis L.* extracts. *Anticancer Res.* 2001 21(5): 3331-9

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Motohashi N, Wakabayashi H, Kurihara T, Takada Y, Maruyama S, Sakagami H, Nakashima H, Tani S, Shirataki Y, Kawase M, **Wolfart K**, Molnar J. Cytotoxic and multidrug resistance reversal activity of a vegetable, 'Anastasia Red', a variety of sweet pepper. *Phytother Res.* 2003 Apr 17(4): 348-52

ABBREVIATIONS

ABC	ATP-binding cassette
AMP	ampicillin
Asp	aspartate
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
CAT	chloramphenicol-acetyl-transferaze
CCW	counterclockwise
CW	clockwise
DHFR	dihydrofolate-reductase
DNA	Deoxyribonucleic acid
ER	erythromycin
ESBL	extended spectra β-lactamase
FACS	fluorescence analyzer cell sorter
FAR	fluorescence activity ratio
FIC	fractional inhibitory concentration
FIX	fractional inhibitory index
Hfr	high frequency of resistance
kb	kilobasis
LRP.	lung resistance-related protein
MATE	multidrug and toxic compound extrusion (family)
MDR	multidrug resistance
MIC	minimal inhibiting concentration
MFP	membrane fusion protein
MFS	major facilitator superfamily
MRP	multidrug resistance-associated protein
MRSA	methicillin-resistant *S. aureus*
MTT	methyl-tetrazolium-bromid
MTY	tryptone-yeast extract media
MVP	major vault protein
NBD	nucleotide binding domain

OD	optical density
OMP	omeprasol
PABA	para-amino-bensoe-acid
PAR	parent
PBP	penicillin binding protein
pBR	plasmid Bolivar-Rodriguez
PBS	phosphate buffered saline
P-gp	phospho-glycoprotein
PK	protein kinase
PMF	proton motive forces
PMZ	promethazine
Qac	quaternary ammonium compound
R123	rhodamine 123
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
RND	resistance-nodulation-cell division (family)
R plasmid	resistance plasmid
S	svedberg
SMR	small multidrug resistance (family)
TEP	telomerase-associated protein
TET	tetracycline
TF	trifluoro-methylketones
TMS	transmembrane segment
UV	ultraviolet
YTB	yeast extract –tryptone broth
VP	verapamil
VRSA	vancomycin-resistant *S. aureus*

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1. INTRODUCTION

1.1 Antibiotic resistance in bacteria

1.1.1. Epidemiology of resistance

Since the introduction of penicillin into the clinical practice in 1938 [1], the antibiotic era had undergone big change. It means that the originally effective antibiotics had become step by step ineffective against certain bacteria of clinically importance, which leads to therapeutic failures. This phenomenon can be due to several causes: first of all the very wide spread application of penicillin from the fourties which had followed to the appearance of the first penicillin resistant *S. aureus* strain [2]. On the other hand, the inappropriate application of further more effective antibiotics: e.g. unwarranted prescribing of antibiotics by physicians and too short–time taking of medicines by patients, can cause the selection of bacterial strain having more moderate sensitivity against the drugs [3, 4, 5]. The insufficient hygienic conditions in hospitals, particularly on intensive care units, had lead also to appearance and spread of poliresistant bacteria [6]. Finally the antibiotic resistance can be due to the marvellous adaptable ability of bacteria to the changed environment, which ability can be due to their very plastic genetic stock [7, 8].

1.1.2. Definition of drug resistance

After the introduction of antibiotics into chemotherapy resistant bacteria were rapidly appeared due to acquiring genes coding for some resistance mechanism. However not all bacteria are intrinsically sensitive to all antibiotics. This type of "resistance" is called **intrinsic resistance** or bacteria with this phenomenon are termed insensitive. This phenomenon is a natural property of a certain bacterial strain and is predictable in clinical situation [9,10,11]

However the so-called **acquired resistance** is occurred among bacteria which are originally sensitive to a certain antibiotic. Two types of acquired resistance can be mentionable: in the case of *mutational resistance* in a large population of bacterial cells a very few individual cells may spontaneously become resistant to one certain drug and the long-term administration of this drug can select the resistant cells. In *transmissible resistance*, genes conferring antibiotic resistance are transferred from a resistant strain to a sensitive one. Exponential transfer and spread of existing resistance genes through a previously sensitive

bacterial population is a much efficient mechanism than is the development of resistance by spontaneous mutation, however the selective pressure by the presence of appropriate antibiotics is also needed to genes becoming widespread. The mechanisms of different gene transfers are discussed later [10,12,13]

1.1.3. The genetic of resistance

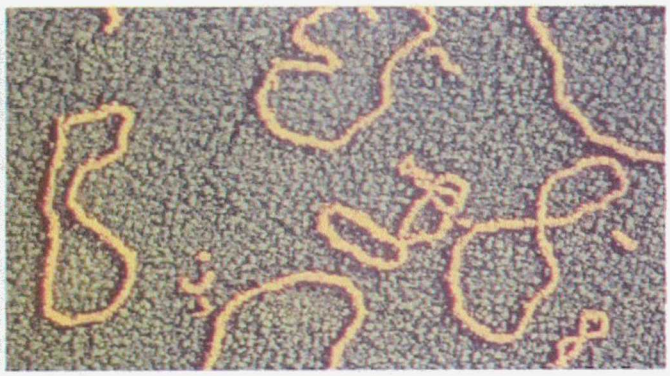
All the properties of a microbial cell are determined by the microbial genome, which comprises the three sources of genetic information in the cell: the chromosome, plasmids and bacteriophages.

1.1.3.1. The bacterial chromosome

Each bacterial cell has a single chromosome which is known to form a single closed circular DNA molecule. In *Escherichia coli* this single DNA comprises about 4 x 10^3 kb (kilobases), is about 1,4 mm in length and encode for 1000-3000 genes. The chromosome is coiled and looped into a compact bundle in the cytoplasm and is not separated from the cytoplasm by any form of nuclear membrane. Transcription of DNA and translation of RNA can therefore proceed simultaneously ensuring very rapid synthesis of proteins which serves rapid accommodation for bacteria to changed environment [10, 14]

1.1.3.2. The bacterial plasmids

The bacterial chromosome carries all the genes necessary for the survival and replication of the bacterial cell under most circumstances. Most of the bacteria also carry additional molecules of DNA (2–200 kb) known as plasmids, which are separate from, and normally replicate independently of, the bacterial chromosome (Figure 1.). Plasmids carry genes which confer a wide range of properties that are *not* essential for the survival of the cell under normal circumstances but provide survival advantage in unusual or adverse conditions. Many different kinds of plasmids have been described depending on the properties what they determine: *Col* plasmids carry genes for bacteriocins, bacterial proteins which can destroy other bacteria [15,16]. *Virulance* plasmids encode genes for toxins, adhesins and other proteins that allow the bacteria to establish infection and cause disease. The metabolic plasmids carry genes for unique metabolic properties of some bacteria. For instance nitrogen fixation by Rhizobium, or the F' lac plasmid in *E. coli* which encodes the lactose permease enzyme for metabolization of lactose [17,18,19,20]



Plasmids are small circles of DNA found naturally in the cells of some organisms. A plasmid can replicate itself as well as any other DNA inserted into it. For this reason, plasmids make excellent cloning vectors—structures that carry DNA from cells of one species into the cells of another.

Figure 1. Plasmids as small circles of DNA

(www. Universe-review.ca/I10-71-plasmid.jpg)

1.1.3.3. The spread of antibiotic resistance genes [21]

The antibiotic resistance genes could horizontally spread by transformation, transduction, conjugation and also by artificially recombinant DNA technology.

In the course of **transformation** DNA fragments from a dead degraded bacterium or from artificially dispatching bind to DNA binding proteins on the surface of the competent recipient bacterium. Nuclease enzymes cut the bound DNA into smaller fragments. One strand of DNA is destroyed, the other strand penetrate the recipient bacterium. This donor DNA fragment is the exchanged for a piece of DNA fragments deriving from recipient by means of Rec A proteins [22, 23].

Transduction is DNA transfer transmitted by bacteriophages. In the lytic phase the virulent phage injects its nucleic acid content into bacterium, the genes of bacteriophage are expressed using the energy and enzymes of host cell, the synthesised elements of virus assemble and the virions release from host cell which is solved by this process. However the so-called temperated phages also injects their nucleic acid into host bacterium, but do not induce lytic cycle, but the phage-DNA will be a part of bacterial genome by integration into bacterial chromosome or only circularise and works as a plasmid. This is the lysogenic phase. For certain stimulation e.g. shock, UV-light this prophage can be activated and the lytic cycle takes place. When integrated phage-DNA cut out of bacterial chromosome some host genes can be withcut and will be a part of phage genom and by a next phage infection these genes of non-phage origin can be horizontally transferred into a new host bacterial cell. In the lysogenic phase the new genes are expressed altering the property of host cell. [24, 25]

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Conjugation is transmission of genes from a living donor bacterium to a recipient bacterium by the means of plasmids. The donor property is possessed on an extra chromosomal elements, on the so-called F^+ plasmid, coding only for the sex pilus (in gram-negative bacteria). The sex pilus binds to the recipient, then a plasma bridge is formed and after breaking F^+ plasmid at *ori* T locus, one strand of F^+ plasmid gets through the plasma bridge to the recipient, the other strand remains in the donor. Each strand then makes a complementary (transfer replication) and the recipient becomes an F^+ donor. There is possibility to transfer chromosomal genes from Hfr donor strains to recipients. The F^+ plasmid becomes linear and integrates into the determined place of chromosome. The linear plasmid still works as an F^+ factor, it breaks at the *ori* T locus, forms sex pilus and plasma bridge and because of the integration the plasmid-chromosome complex gets through the plasma bridge to the recipient. The bacterial connection usually breaks before the transfer of the entire chromosome is completed so the reminder of F^+ plasmid seldom enters the recipient. As a result there is a transfer of some chromosomal DNA from donor to recipient. [26, 27, 28]

Other plasmids like resistance plasmids (R-plasmid) coding for multiple antibiotic resistance and sex pilus formation are able to get to the sensitive recipient by the same process and the recipient becomes multidrug resistant and F^+ so will be able to transfer R-plasmids to other sensitive strains.

1.1.4. The mechanisms of antibiotic resistance

The antibiotic resistance involves severe mechanisms and the situation of genes coding for the certain resistance can be also very diverse. The resistance mechanisms are the further: enzymatic inactivation of antibiotics, enzymatic modification of antibiotics, alteration of drug targets, bypass mechanisms which are alternative ways of the original biochemical processes to be inhibited, changing of permeability and active efflux mechanisms [29]

1.1.4.1. Enzymatic inactivation of antibiotics

In the case of β-lactam antibiotics the bacterial β-lactamases can destroy the drug by creating an enzyme-drug complex, then the β-lactam-ring splits with the inactivation of

molecule and regeneration of the enzyme. The Gram- negative bacteria show wide range of β-lactamases depending on the localization of genes, ability of induction, range of spectra. According to the Bush – Livermore classification the **"class C"** –type enzymes (amp C) are the most general among *Enterobacteriaceae*, are coded mostly on the bacterial chromosome, plasmids and integrons, and strains producing of this enzyme at high level can cause serious nosocomial infections due to selection mechanisms [30,31,32]. The **"class A"**-type enzymes (K1, KOXY) are produced mostly by *Klebsiella* spp, coded also on the bacterial chromosome and their overproduction causes resistance of high level to β-lactam antibiotics except for carbapenems and ceftazidime. **"class B"**-type enzymes (L-1, L-2) are metalo-β-lactamases, coded chromosomally and cause resistance to each β-lactam antibiotics including carbapenems in non-fermentative Gram- negative rods [33,34,35]. The so-called extended-spectra-β-lactamases **(ESBL)** are coded on plasmid, the TEM-1,-2, SHV-1, OXA-1 enzymes belong to this group. Among β-lactam antibiotics only carbapenems and cefamycins are effective on bacteria producing these enzymes. Since the resistance genes are coded on plasmid, these bacteria – mostly *Klebsiella* spp.- can easily transfer multiple antibiotic resistance to other bacterial strains via the process of conjugation causing dangerous nosocomial epidemic [36,37,38].

Macrolid antibiotics can be also inactivated by specific enzymes coded usually on plasmids.

1.1.4.2. Enzymatic modification of antibiotics

Amynoglycosides are neutralized by enzymes e.g. acetyl-transferaze, phosphotransferaze and nucleotidil-transferaze by connecting the certain groups into the molecules which become ineffective [39,40]. These enzymes are coded chromosomally or on plasmids. The **chloramphenicol** can be modified by chloramphenicol-acetyl-transferaze (CAT) [41,42].

1.1.4.3. Alteration of drug target

In cell wall synthesis the bacterial penicillin binding proteins (PBP) play a major role. During synthesis they bind the cell wall precursor, but the **β-lactam** antibiotics as well due to a structural similarity. In the last case the mechanism of cell wall synthesis destroys and the bacterium solves. However the structure of PBPs change without losing the ability to act normally at cell wall synthesis, but the antibiotics cannot bind PBP so β-lactams will be ineffective. Genes coding for the changed PBPs are usually derived from another species and

due to transformation and later recombination these genes have so-called mosaic-structure [29,43,44]

In **amynoglycosid** resistance the bacterial 30 S ribosome alters as a target of antibiotic and in **macrolid** resistance the 50 S ribosomal subunit modifies with alteration of its rRNA base sequence. In tetracycline resistance the ribosome changes also by producing a protein which protects it from before antibiotic [45,46,47].

In the resistance mechanisms of **glycopeptides** the end of a protein involving in cell wall synthesis, as a structure-terminal-pentapeptid, alters from D-alanil-D-Ala to D-alanil-D-laktate which cannot be bound by glycopeptides, but it still works normally in synthesis. Genes coding for this kind of resistance mechanisms are often transferred from glycopeptide resistant Enterococci to MRSA (methicillin-resistance *S. aureus*) producing the so-called VRSA (vancomycin-resistant *S. aureus*) which bacterium is a threatening participant of bacterial infections [48,49,50].

1.1.4.4. Bypass mechanisms

When a biochemical process to be originally inhibited by antibiotics goes through in an alternative way, antibiotics won't be able to influence it. It happens in the case of classical methicillin resistance of *S. aureus* which create an extra PBP, so-called PBP2a, which cannot be bound by any β-lactam. The MRSA strains are resistant to each β-lactam antibiotics and bear cross-resistance to some other antibiotic classes. Extra PBP 5 is produced in *E. faecium* as well meaning its ampicillin-resistance [49,51]. In sumetrolim and in trimethoprim resistance two bacterial biochemical intermediate product to be inhibited by antibiotics are overproduced, the PABA (para-amino-bensoe-acid) and DHFR (dihydrofolate-reductase) respectively. Genes coding for this resistance usually are localized on some mobile genetic elements e.g. plasmids, transpozones [29].

1.1.4.5. Changing of permeability

In Gram-negative bacteria the outer membrane contains the so-called protein porin-channels which ensure the enter of antibiotics and other unrelated compounds into the cell. Due to mutations decreasing the number of porins or changing the structure of porins follow to the increasing MIC values and/or resistance of beta-lactam antibiotics particularly of carbapenems. This happens in the case of isolated carbapenem-resistance of *Enterobacteriaceae* or isolated imipenem-resistance of *P. aeruginosa* which preserves sensitivity to meropenem [29,52,53,54].

1.1.4.6. Active efflux

The drug efflux can be realised through the working of bacterial drug transporters. Some transporters, such as the tetracycline efflux proteins (Tet), are dedicated systems which mediate the extrusion of only a given drug or class of drugs [55,56]. In contrast to these specific drug transporters, the so-called multidrug transporters can handle a wide variety of structurally unrelated compounds and can be divided into two major classes. Secondary multidrug transporters utilize the transmembrane electrochemical gradient of protons or sodium ions to drive the extrusion of drugs from the cell, ATP-binding cassette (ABC-type) multidrug transporters use the free energy of ATP hydrolysis to pump drugs out of the cell [55, 57, 58, 59].

1.1.4.6.1. Secondary multidrug transporters

These transporters mediate the extrusion of toxic compounds from the cells in a coupled exchange with protons. On the basis of size and similarities in the primary and secondary structure, they can be divided into four distinct families of transport proteins.

1.1.4.6.1.1. major facilitator superfamily (MFS)

It consists of membrane transport proteins which can be divided into two separate clusters with either 12 or 14 transmembrane segments (TMS) [60] (Figure 2).

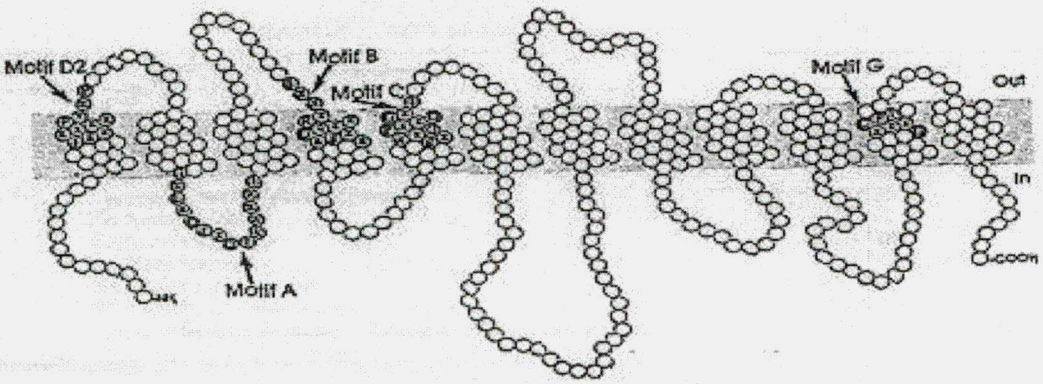


Fig 2. Structural model for the 12-TMS multidrug transporters of the MFS. Conserved sequences are shaded.

In the 12 TMS class the *S. aureus* NorA (norfloxacin) protein was first discovered in quinolone- and methicillin-resistant clinical isolate [61,62] and two proteins, Bmr and Blt of *B. subtilis* can be mentionable [63,64].

The 14 TMS class of MFS comprises among others the QuacA and QacB (quaternary ammonium compounds) proteins which play a major role in the resistance to antiseptic and

disinfectant compounds among multidrug-resistant *S. aureus* by pumping out cationic dyes/ membrane-permeable organic cations and quaternary ammonium disinfectants [65,66]

1.1.4.6.1.2.small multidrug resistance (SMR) family

The smallest secondary drug efflux proteins known consist of tightly packed four-helix antiparallel bundle and may function as homooligomeric complexes due to the small size [67].

The firs gene (*qacD*) encoding a transporter protein of the SMR was detected on both conjugative and nonconjugative plasmids from clinical isolates of *S. aureus* [68,69,70]. Among gram-negative bacteria among others the *E. coli* multidrug transporter, EmrE (MvrC) has been identified. It is situated in the inner membrane layer and the drugs appear to be exported into the periplasmic space. Overproduction of the protein makes *E. coli* slightly more resistant to tetracycline, erythromycin and sulfadiazine. The pumps of Smr type, however, are not known to make significant contributions to clinically relevant resistance among gram-negative bacteria [71,72].

1.1.4.6.1.3. multidrug and toxic compound extrusion (MATE) family

It contain 12 putative TMS similarly to MFS but do not have sequence similarity with any member of MFS. NorM in *V. parahaemolyticus* and YdhE in *E. coli* belong to this group mediating resistance to dyes, hydrophilic fluoroquinilones and aminoglycosides. The third distinct cluster hypothetically comprises multidrug efflux proteins from *H. influanzae* [73,74].

In gram-negative bacteria the former transporters situated in the inner layer of plasma membrane cannot be effective enough since the lipophilic compounds can spontaneously diffuse back from the periplasmic space into the cytoplasm. More complex systems were needed for clinically relevant drug resistance for gram-negative bacteria.

1.1.4.6.1.4. The Resistance Nodulation Cell Division family (RND)

Multidrug transporters belonging to the RND family are located in the cytoplasmic membrane; interact with a periplasmic membrane fusion protein (MFP) and an outer membrane channel protein (Figure 3.) to allow drug transport across both the inner and the outer membrane of gram-negative bacteria. Because these pump systems can excrete drug molecules directly into the medium, they can produce significant resistance levels [75,76].

1.1.4.6.1.4.1. Structure of RND-type transporters

The secondary structure of RND-type transporters /efflux proteins consist of 12 TMS α-helix with two large loops between TMS1 and 2 and TMS 7 and 8 (Figure 3.) [77]. Like any other secondary transporters it also uses proton motive forces (PMF) for the active efflux of compounds.

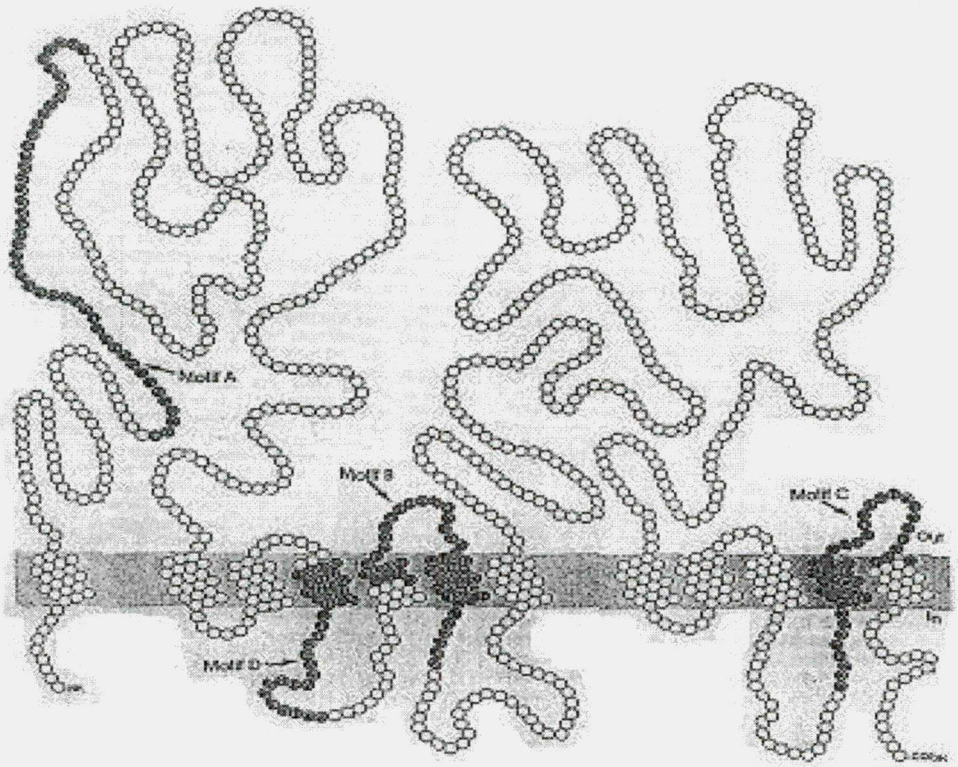


Fig. 3. Structural model for the multidrug transporters of the RND family. The shaded conserved motifs on Figure 2,3 are charged amino acid residues in TMS which is energetically unfavourable suggesting important structural and functional role in the transporters. **Motif A** may be involved in reversible conformational change required for the opening / closing of the transport channel. **Motif B** is responsible for proton transfer. **Motif C** dictates the direction of transport, that's why isn't found in symporters of MFS.

1.1.4.6.1.4.2. Structure and role of AcrAB-TolC efflux pump

One of the main representatives of this type of pumps and one of the main participant of this thesis is the so-called **AcrAB-TolC** efflux pump system in *E.coli* (Figure 4.). Acr B is the RND-type transporter, AcrA is the periplasmic membrane fusion linker protein (MFP), which links the inner membrane (containing the AcrB transporter) and the outer membrane channel protein, TolC, together. This is reinforced by the finding that the member of MFP have some homology with a paramyxovirus membrane fusion proteins [75,72].

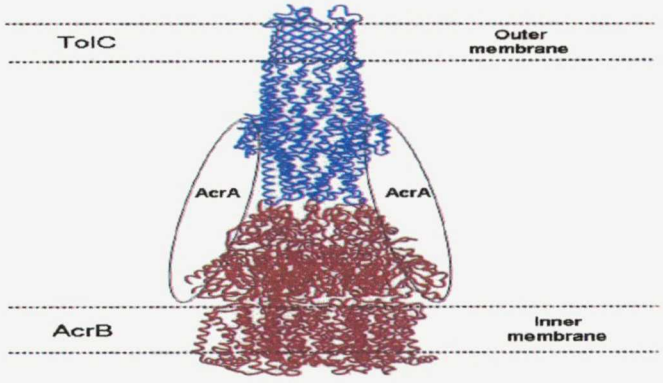


Figure 4. The structure of AcrB-TolC efflux pump system
(www.indigo1.biop.ox.ac.uk/loredana/acrb.html)

It is well known that activator proteins are produced at a higher level in response to the presence of antibiotics, superoxid radicals and these regulator proteins are known to increase the efflux of several drugs [78,79,80]. In addition to the increased transcription of the acrAB operon containing the genes coding for proteins of AcrAB pump, the activator proteins down-regulate the synthesis of the major porin OmpF. The decreased outer membrane permeability thus synergistically enhances the effect of an increased production of the efflux machinery [79].

1.1.4.6.1.4.3. Substrates of AcrAB-TolC efflux pump, mechanism of drug efflux

AcrAB system pumps out an extraordinary wide variety of antibiotics, chemotherapeutic agents, detergents and dyes. Many of the substrate carries net negative charge, but there are also compounds with positive charges, such as cationic dyes and erythromycin. The common characteristics among substrates can be glimpsed by examining drugs that are *not* exported by this system. There is no evidence for the efflux of the very hydrophilic aminoglycosides [81,82]. It is evidence that AcrAB system can produce significant resistance to large hydrophobic agents e.g. erythromycin which was studied in the experiments involved in this thesis as well [83,84]. Most of the natural antibiotics belong to this class and their range has traditionally been considered to be limited to gram-positive bacteria. The intrinsic "gram-negative-resistance" is often caused by the efflux pumps of the whole RND family [71,85] and efflux work in synergy with the outer membrane barrier [79].

The multidrug transporters transport their hydrophobic substrate from the lipid bilayer rather than from cytoplasmic aqueous phase. The only requirement for AcrAB pump is that the hydrophobic substrate becomes inserted into the lipid bilayer of the membrane. It is proposed that the substrates are captured from within the bilayer [75,86,87].

1.1.4.6.1.4.4. Homologues of AcrAB-TolC efflux pump

The AcrAB and its homologues are apparently widespread among gram-negative bacteria. Among *Pseudomonas aeeruginosa* strains among others the MexAB-OprM, MecCD-OprJ and MexEF-OprN [88,89,90]. Further examples include MtrCDE in *Neisseria gonorrhoeae* [91] and a homolog found in *Haemophilus influenzae* [92]. A homolog of the outer membrane channel TolC was also found in *Burkholderia cepacia* [93].

1.1.4.6.1.5. The normal physiological function of multidrug transporters

The drug-efflux pumps are certainly not a recent product of the antibiotic age. The normal physiological function of multidrug transporters with special regard to the RND-type pumps is to play a role in natural defence mechanisms of bacteria against toxic compounds that exist in the environment. First lots of gram-negative bacteria live in a habitat with high concentration of lipophilic inhibitors, e.g., *E. coli* is faced with bile salts and fatty acids in the intestinal tract. AcrAB was shown to play role in the efflux of bile salts allowing *E. coli* to survive in such environment [94]. Similarly *N. gonorrhoeae* strains isolated from the rectum frequently overproduce the MtrCDE pump [91] which allows survival in an environment full of lipophilic inhibitors [95]. Second, the extremely wide substrate specificity of these pumps will be best suited for this defensive role, as the bacterium cannot predict the nature of the inhibitor it will face. Third, the AcrAB pump appears to be regulated more by global stress signals rather than by presence of specific substrates [80]. Fourth endogenous substrates have not been found for any of these pumps [71].

Genes determining multidrug resistant efflux pumps are mostly situated on bacterial chromosome but there are any reasons to fear that these genes can become a part of R plasmids, because divalent cation efflux pumps that contain the three components of the RND-family [75,76] are already known on plasmids [96], thus, R plasmids may indeed acquire the genes for multidrug efflux pumps in the future.

1.1.4.6.2. ATP-dependent multidrug transporters

Although most bacterial multidrug transporters utilize the PMF (proton motive forces) (or sodium) for the extrusion of cytotoxic compounds, some drug efflux systems are driven by free energy of ATP hydrolysis. All ATP-dependent drug efflux proteins known to date are members of the ABC (ATP-binding cassette) superfamily.

1.1.4.6.2.1. Structure of ABC transpsorters

ABC transporters require four distinct domains: two highly hydrophobic (N-terminal) membrane domains, which usually consist of six putative transmembrane α-helices each, and two hydrophilic (C-terminal) nucleotide binding domains (NBDs), containing the Walker A and B motifs [97] and the ABC signature [98] (Figure 5.). The individual domains can be expressed as separate proteins or may be fused into multidomain polypeptides [98,99].

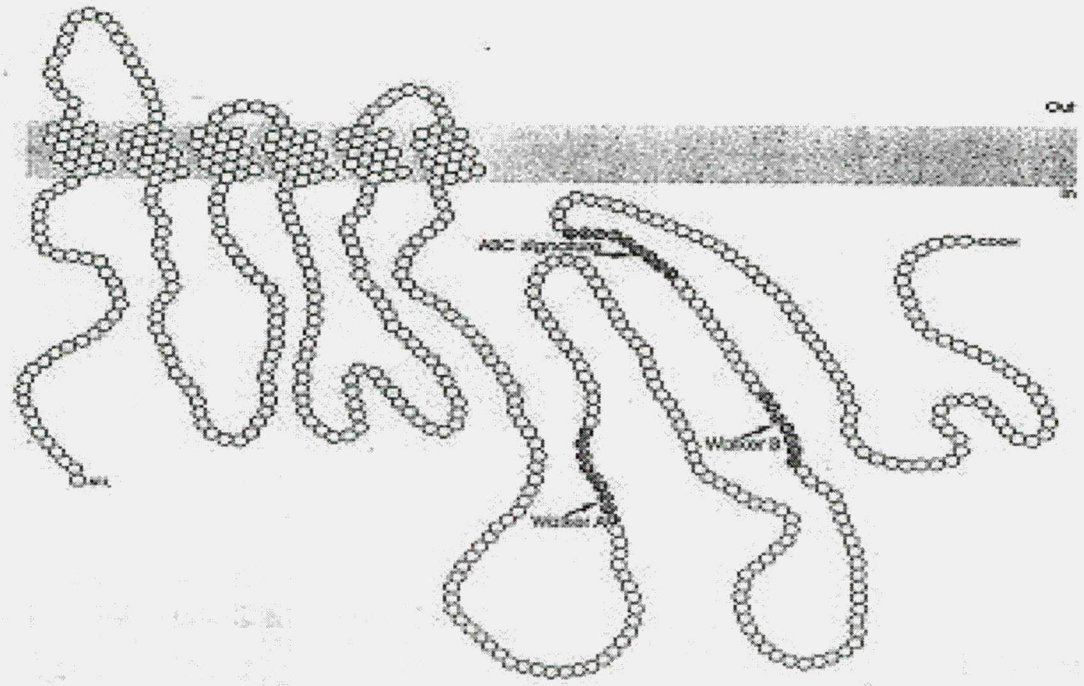


Figure 5. Structural model for multidrug transporters of the ABC superfamily with Walker A and B motifs and with ABC signature sequence which are shaded. In view of the general four-domain organization, it may function as a homodimer.

1.1.4.6.2.2. Homologues among ABC transporters: bacterial LmrA, haemolysin transporter and human P-glycoprotein

Most bacterial ABC drug transporters mediate the export of specific antibiotics [100,101,102]. The firs true bacterial ABC multidrug transporter was found in *Lactococcus lactis* [103] and the gene encoding this transporter, designated *lmrA*. LmrA protein is homologous to each of the two halves of the human multidrug transorter P-glycoprotein (P-gp) (see below) [104] and these two transporters are functionally interchangeable, indicating, that this type of multidrug transporter is conserved from bacteria to humans [105]: genome sequencing revealed the presence of putative LmrA homologues in *E. coli*, *B. subtilis*, and the pathogens *Helicobacter pylori*, *Mycobacterium genitalium*, *H. influenzae* and *S. aureus*

[106]. It is common, that TMS 5,6,11 and 12, and the loop between TMS 2 and 3, and TMS 11 and 12 are most important in drug binding and/or transport [107,108,109]. Moreover LmrA and P-gp includes parts of TMS 5 and 6 and the loop between TMS 2 and 3 [104], indicating that these particular regions may be important for substrate binding in both human and bacterial ABC-type multidrug transporters. The HlyB transporter of *E. coli* is involved in transport of bacterial haemolysin and has also a structural similarity with the human P-gp. Both have channel-forming functions that act like a flippase (an enzyme which translocate lipids in membranes between the two bilayers). The extent of the protein sequence homology between P-gp and HlyB transporters suggests that these two proteins share a common function in the plasma membrane, exporting molecules from the cells [110]. Moreover drugs of similar chemical structure e.g. certain type of phenotiazines can inhibit both transporters indicating their structural and functional similarity [111].

1.2. Resistance mechanisms in tumour cells

In cancer therapy the tumour cells may demonstrate single agent resistance or resistance to a single class of anti-cancer drugs with the same mechanism of action. This can be overcome by addition of different classes of drugs to a chemotherapy regimen. However there may be cells which are broadly resistant to many chemically diverse anti-cancer drugs with different mechanisms of action. The latter phenomenon is the multidrug resistance (MDR) and is a more urgent problem than former one [112]. Several different resistance mechanisms cause MDR e.g. interference with programmed cell death following treatment with cytostatics by inactivation of p53 [113], resistance-related abnormalities of enzyme topoisomerase II [114,115], overexpression of genes involved in the glutathione S-transfer system [116,117], and expression of energy-dependent pump systems which either exclude or extrude anti-cancer agents from the cells.

1.2.1. ABC transporters in tumour cells

One of the largest protein classes known, the ABC (ATP-binding cassettes) transporters (Figure 5.) using energy deriving from ATP hydrolysis for pumping the drugs out of the cells, are involved in most cases of this mechanisms [118,119,120]. Three major groups of ABC transporters may play a role in tumour's intrinsic and/or acquired resistance:

*1.2.1.1. The **multidrug resistance-associated protein (MRP** or ABCB1) group*

It involves seven member e.g. MRP 1-7 (or ABCC1-6 and ABCC10) and consists of two homologous halves each containing six transmembrane regions and one ATP binding / utilization site (see below structure of p-gp) [112,121]. MRPs are (multispecific) organic anion transporters, which can transport water-soluble negatively charged anionic drugs and hydrophobic uncharged molecules as well. Transport of neutral drugs is carried out by their conjugation to glutathione, glucuronate or sulphate. MRP4 and MRP5 broaden the spectrum of drug resistance to nucleotide analogue drugs [121,122]

*1.2.1.2. The **breast cancer resistance protein (BCRP)** (also known as MXR, ABCP, ABCG2)*

This is a novel member of ABC transporters. The termination is due to the fact that it was recently discovered at first time in MCF-7/AdrVp human breast carcinoma cell line. The protein consists of 655 amino acids, altogether is about 72 kDa and maps to chromosome 4q22. BCRP belongs to the ABC G subfamily and is structurally a so-called half-transporter [120,123]. It forms a dimer to produce an active transport complex. In contrast to MRP, BCRP preferentially extrude large *hydrophobic, positively* charged molecules [122,123,124]

*1.2.1.3. **P-glycoprotein***

The third major member of ABC transporters is the **MDR1 or p-glycoprotein**. A protein, which is one of the main causes of clinical MDR in tumour cells and which is the most studied transporter protein in MDR reversing experiments. /Details see below/

1.2.2. Lung resistance-related protein (LRP)

The further protein is *not* a member of ABC transporters but is also a transport-associated protein termed the **lung resistance-related protein (LRP)**. LRP was identified as the major vault protein (MVP), the main component of multimeric vault particles. With the recent identification of the two minor vault proteins as telomerase-associated protein (TEP1) and vault-poly (ADP-ribose) polymerase (VPARP), the composition of vaults is almost unravelled [125]. Vaults are novel cellular organelles broadly distributed and highly conserved among diverse eukaryotic cells, suggesting they play a fundamental role in cell life. Since the localization of vaults is in the nuclear pore complexes, nucleocytoplasmic transport of several diverse drugs by vaults may be supported causing multidrug resistance. There is direct evidence for a casual relationship between LRP/MVP expression and drug resistance. LRP is widely distributed in clinical cancer specimens, but the frequency of LRP

expression inversely correlates with the known chemosensitivity, so its expression at diagnosis predicts poor prognosis. [125,126]

1.2.3. The structure and role of human P-glycoprotein / MDR1

The 170 kDa transporter protein (p-gp 170) may be the major factor of cancer cells' multidrug resistance. It belongs to the superfamily of ABC transporters (see above) (Figure 5.). The *mdr1* gene, a so-called SOS gene, involved in p-gp 170 protein production is localized in human chromosome 7 band p 21,0 – 21,1 [127]. P-gp is 1280 amino acids in length. The primary amino acid sequence predicts a protein with 12 transmembrane domains in two homologous halves, each containing six transmembrane regions with 43 % amino acid-sequence homology between the amino- and carboxy-terminal halves, and two large intracytoplasmic loops encoding an ATP-binding and utilization sites (also called nucleotide binding sites / NMSs) [128,129,130] (Figure 6.).

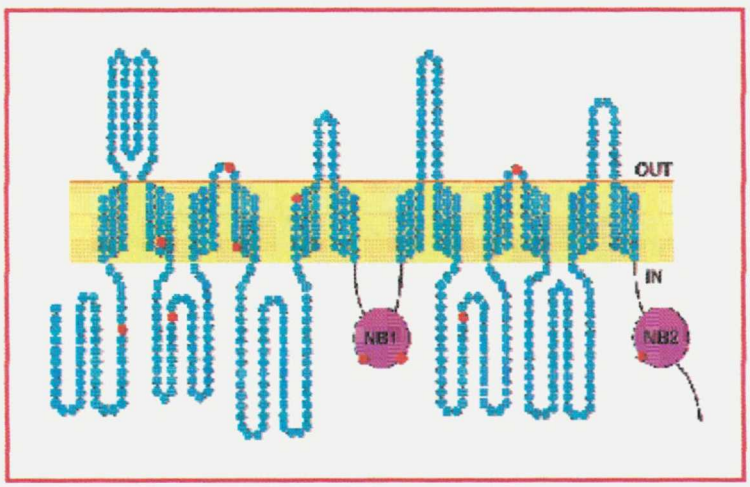


Figure 6.. The structure of P-glycoprotein 170 molecule with two nucleotide binding domain (NB1 and NB2)

(www.uoguelph.ca/~fsharom/research/images/trp_topology_on_white.gif)

1.2.3.1. Substrates and operation of human P-glycoprotein

The mdr-1-encoded P-glycoprotein pump recognizes and transports out of the cells many different substrates, including most natural product anti-cancer drugs such as doxorubicin, daunorubicin, vinblastine, vincristine, actinomycin D, paclitaxel, teniposide and etoposide which only share the properties of being hydrophobic amphipathic molecules which are not negatively charged (opposite to MRP) [131]. However it may thus able to recognize negatively charged hydrophilic compounds if they enter the cell by passive diffusion and become accessible to the pump from the membrane environment [132,133].

Consideration of the extremely broad range of substrates, two possible models for the transport action exist: first, the " altered partitioning" model envisions that overexpression of p-glycoprotein leads to an alteration of the cell's electrical membrane potential and / or an elevated intracellular pH. These perturbations then either directly or indirectly alter the partitioning and intracellular drug accumulation and the electrochemical and / or H+ gradient acts to drive the movement of charged drugs across the membrane. In this model the p-gp itself is not a drug transporter, but has an indirect role in modifying the intracellular environment [134,135]. Second, in the "pump model", the energy of ATP hydrolysis by p-glycoprotein is utilized for the removal of drugs from cell membranes and cytoplasm analogous to the ion-translocating pump. The pump recognizes substrates directly through a complex substrate recognition region. In this model the p-gp does work as a drug transporter (efflux pump) [136,137,138]

The majority of the published data strongly favours the second model, as evidence for a direct interaction of many of the substrates with the transporter has been obtained including drug binding sites on p-gp, drug-stimulated ATPase activity in direct proportion to the ability of p-gp to transport these drugs. Arguments for the "altered partitioning" model are supposed by the finding of increased cytoplasmic pH and altered membrane potential in some cells that express p-glycoprotein [134,139]. However evidences argue that these electrochemical gradients are not in themselves sufficient to account for the high level of drug resistance seen in many multidrug resistance cells expressing p-glycoprotein [112].

There seems no doubt that substrates for transport by p-gp interact directly with the transporter – probably in the region of transmembrane segments 5,6 and 11,12 [140,141]. It has proposed that p-glycoprotein is a "hydrophobic vacuum cleaner" or a "flippase" [142,143] as the anticancer drugs enter the plasma membrane and the drugs with a hydrophobic and a positively charged domain demonstrate diffusion into and across the plasma membrane, where encounter the multidrug transporter by lateral diffusion. The transporter uses energy transduced from two essential ATP binding sites to pump the drug out of the membrane [144]. The p-glycoprotein itself catalyzes substrate-stimulated ATP hydrolysis at a rate similar to other ion-translocating ATPases [130], and although both NBSs (nucleotide binding sites) of p-gp bind ATP, and the interaction of both sites is required for ATP hydrolysis, only one site at a given time acts as a catalytic site and the confirmation of this site prevents the other one from hydrolyzing ATP [145,146].

1.2.3.2. Post-translational modifications of human P-glycoprotein

P-gp is post-trsanslationally modified by glycolization and phosphorylation. Experiments show that glycosilation of p-gp is not required for its multidrug transport function, however it has been suggested that N-glycosilation may contribute to the correct folding, proper routing and stabilization of the molecule [147,148,149].

P-gp has five sites which can be phosphorylated on serine and threonine residues by protein kinase (PK) C, PKA or another p-gp-specific kinase at positions situated in the linker region of the molecule [150,151, 152]. Evidences are supported that phorbol esters which activate PKC, might be attributable only to the stimulation of transcription of the p-glycoprotein gene and not to the increasing of drug resistance [153,154].

1.2.3.3. Normal physiological function of human P-glycoprotein

The P-glycoprotein is expressing not only in cancer cells, however a plenty of human tissues express this protein with normal physiological function. Expression was found on luminal surfaces of epithelial cells of the kidney proximal tubules, small and large intestine, and biliary hepatocytes, suggesting a normal function for the excretion of endogenous and exogenous hydrophobic, amphipathic toxins. Expression on capillary endothelial cells of the brain and testis and in the placenta suggested a 'barrier' function to keep toxic materials out of the brain, gonads and foetus. High level expression of P-gp in the adrenal cortex, pancreatic ductules and in steroid-producing endometrial glands in the pregnant uterus have suggested a role in transporting hormones such as aldosterone, dexamethasone, cortisol and corticosterone [155] and in the handling of steroids-perhaps providing a 'protective' function for the plasma membranes of steroid producing cells. Lower expression of P-gp was found in bone marrow stem cells [155,156,157]. Several studies have demonstrated increases in RNA levels for P-gp after partial hepatectomy, treatment with chemotherapeutic drugs and cytotoxic stress such as heat shock [158,159].

1.2.3.4. Presence of P-glycoprotein in malignant tumours

The process of malignant transformation in cancer derived from tissues that do not normally express P-gp can activate expression of the *mdr1* gene [160]. Increased expression of the *mdr1* gene is commonly seen in tumours treated with chemotherapy that have relapsed during the course of, or after chemotherapy like in the cases of breast cancer, ovarian cancer, lymphoma, leukaemia, neuroblastoma, pheochromocytoma, rhabdomyosarcoma and multiple myeloma. In these cases it is presumed that small numbers of *mdr1*-expressing cells were present when therapy was initiated and that this population survived chemotherapy and

caused the relapse. But a direct effect of chemotherapy to induce *mdr1* gene expression is also possible [161].

1.3. Possibilities for overcoming drug resistance in bacteria and tumour cells

The main aim of this work –after the introduction of the different type of resistance in bacteria and tumours - is to demonstrate some practical solutions to overcome resistant in bacteria and tumour cells as well. Theoretically decreasing the resistance can be achieved by different ways: in bacteria eliminating the extrachromosomal genetic elements (e.g. plasmids), inhibiting their intercellular transfer in the population and enhancing the uptake of antibiotics, exerting direct effects on drug accumulation of microorganisms and cancer cells as well by either direct inhibition of their efflux transporter proteins.

At the Department of Medical Microbiology at University of Szeged intensive experiments have been carried out since the seventies in the field of reversing drug resistance. First of all some phenotiazines, e.g. chlorpromazine, levopromazine and promethazine were shown to have bacteriostatic and bactericide effect, and the chlorpromazine was effective in eliminating of R factor indicating having some resistance reversal effect [162]. Later further more psychotropic drugs were shown to have antiplasmid effect, since the F'lac plasmid of *E. coli* was successfully eliminated [163] and the mechanism of plasmid curing by surface action of the drugs was suggested as an alternative to direct intercalation of the drugs into plasmid DNA [164]. Later it was found that the guanine-cytosine rich regions of plasmid-DNA is the target in the antiplasmid effect of phenotiazines [165]. Alterations in the bacterial membrane such as discontinuities, phase separation or rarely extensive lytic alterations caused by some phenotiazines, imipramine were observed during elimination experiments and the drugs were proved to bind to two different receptor sites (strong and weak) simultaneously on the plasmid replication site [166,167]. The tricyclic configuration of the psychotropic drugs, which were tested for stereospecific binding to bacterial receptors, was found to be more important than its side chain orientation and similarity is believed between bacterial and neural receptor sites. The stereochemical configuration of the curing agents does influence the complex process of plasmid elimination [168,169]. The tricyclic compounds having antiplasmid effect were divide into three groups according to the numbers (0,1 or 2) of heteroatom in their ring system serving a guideline for drug design in the future to develop related potent substances with less side-effect [170].

However the elimination of antibiotic resistance-encoded plasmids from all individual cells of the population is never complete. Its medical significance could be that elimination method may provide a technique to isolate plasmid-free bacteria for biotechnology without any risk of mutations.

This thesis introduces experiments by which the antibiotic resistance could be reduced in all the cell of the population by the chemical inhibition of the drug efflux pumps in bacteria and tumour cells as well resulting the increased accumulation of antibiotics and cytostatics in bacteria and cancer cells respectively.

SZTE Egyetemi Könyvtár SZEGED 13.

2. AIMS OF THE THESIS

For the rapidly increasing antibiotic resistance urging solutions are needed: either by the introduction of new antibiotics, or by combinations of old antibiotics with resistance modifier agents. In earlier works the reversal of antibiotic resistance meant the elimination of plasmids which confer genes for resistance. Since elimination never occurs in the whole population a more effective mechanism was found for increasing the accumulation of drugs in each cell of the population, namely the inhibition of proton- (in bacteria) and ABC- (in tumours) pumps -extruding wide range of structurally unrelated compounds or xenobiotics out of the bacteria and tumour cells- by different chemical agents.

1. The antibacterial effect of the resistance modifiers such as trifluoro-methylketones, as known proton pump inhibitors (e.g. Omeprasol), other known resistance modifiers eg. verapamil, a phenotiazine e.g. promethazine, were determined on *E. coli* AG100 (operating with proton pump) resistant strain and 100A (proton pump defective) sensitive strain.

2. The most potent trifluoro-methylketones were chosen and examined for interaction in antibacterial effect with promethazine and verapamil in checkerboard microplate method on *E. coli* AG100 and AG100A.

3. A clinically important model was constructed, when *E. coli* AG100 and AG100A were transformed with pBR322 plasmid. These bacteria were considered as clinical model of resistant bacteria. The elimination of pBR322 plasmid by promethazine from the two *E. coli* strains can provide clinical relevance.

4. The most potent trifluoro-methylketone, 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18), was examined in checkerboard method with some representatives of antibiotics (tetracycline, ampicillin, erythromycin) on *E. coli* AG100 and AG100A for inhibitory effect.

5. Since 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) was found to be a potent proton pump inhibitor and resistance modifier, other known proton pump inhibitor (Omeprasol), and 1-(2-benzoxazolyl)-2-propanone (TF51) -a structurally close derivative of TF18- were studied in combination with erythromycin on *E. coli* AG100 and AG100A .

6. Known resistance modifier (promethazine, verapamil) were applied in combination with erythromycin and ampicillin on both *E. coli* strains.

7. As direct evidence for interaction of trifluoro-methylketones with proton motive forces the antimotility action of the compounds was examined on the bacterial motion treating *P. vulgaris* cells.

8. The correlation between the chemical structure of trifluoro-methylketones and their antibacterial, antimotility and proton pump inhibitory effects were studied.

9. In cancer cells, for the possible multidrug resistance reversal effect, the drug accumulation was tested by the Rhodamine-123 extrusion assay. For these experiments

- acridine derivatives: aza mono, bi- and tricyclic compounds
- benzonaphthyridine-5-one derivatives were studied

10. The relationship between the chemical structure of effective agents and the biological effect was evaluated with particular respect to the type of aromatic moiety, and the structure of the side chains (substituents and size)

3. MATERIALS

3.1. Culture media for bacteria

MTY / tryptone-yeast extract media /: liquid media was used for the cultivation of *E. coli* AG 100, AG100A and *P. vulgaris* bacterial strains. /171/

MTY agar: was used for the determination of live cell counts of *E. coli* AG100, AG100A and *P. vulgaris* culture. It was used for the maintenance of the mentioned bacterial strains.

YTB / Yeast extract –Tryptone Broth /: liquid media was used for the cultivation of *E. coli* AG100, AG100A strains to be transformed with pBR322 plasmid and was used for the dilution of bacteria during transformation procedure.

YTB agar /supplemented with tetracycline and ampicillin / was used for the plating, cultivation and selection of *E. coli* AG100 pBR322 and AG100A pBR322 strains.

3.2. Media for cell line cultivation

McCoy's 5A medium modified (GIBCO BRL) /172, 173, 174/

PAR: Streptomycin, Nystatin, 200 mM L-glutamine, 10 % heat inactivated horse serum

MDR: Streptomycin, Nystatin, 200 mM L-glutamine, 10 % heat inactivated horse serum

3.3. Stock solution for preparation of competent cells

Calcium chloride ($CaCl_2$) 1.0 M, Magnesium chloride ($MgCl_2$) 1.0 M, potassium chloride (KCl) 1.0 M

3.4. Dye

3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma)

3.5. Strains and cell lines

3.5.1. Bacterial strains

Laboratory strains: *E. coli* AG 100, *E. coli* AG 100A were constructed and kindly provided by Professor Nikaido (University of California, USA), *E. coli* AG100 pBR322 and *E. coli* AG 100A pBR322: the former two strains transformed with pBR322 plasmid respectively

Clinical isolate: *P. vulgaris* (derived from Institute of Clinical Microbiology, University of Szeged),

3.5.2. Tumour cell lines

(provided by Professor Adorján Aszalós, FDA, Washington, USA)

L5178Y mouse T cell lymphoma cell line (PAR)

L5178 mouse T cell lymphoma MDR 1 / A retrovirus transfected cell line (MDR)

3.6. Compounds studied

3.6.1. Ca channel inhibitors

Promethazine (Pipolphen®) (EGIS Pharmaceutical Company, Budapest, Hungary), verapamil (Verapamil®) (Chinoin, Budapest, Hungary)

3.6.2. Proton pump inhibitor

Omeprazol (AstraZeneca)

3.6.3. Antibiotics

Ampicillin (Penbritin®), erythromycin (Erythromycin lactobionate), tetracycline (Tetracycline®) (Chinoin, Budapest, Hungary)

3.6.4. Trifluoro-methylketones (TF compounds)

TF5: *3-trifluoroacetylindole* (obtained from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan),

TF6: *2-trifluoroacetylbenzoxazole* [175],

TF10: *4,4,4-trifluoro-1-phenyl-1,3-butanedione* [176.],

TF11: *1,1,1-trifluoro-3-(4,5-dimethyloxazol-2-yl)-2-propanone* [176],

TF18: *1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone* [176],

TF19: *3-(2-benzothiazolyl)-1,1,1-trifluoro-2-propanone* [177],

TF20: *3-(2-benzimidazolyl)-1,1,1-trifluoro-2-propanone* [177],

TF50: *3,3,3-trifluoro-2-hydroxypropyl phenyl ketone* [175],

TF51: *1-(2-benzoxazolyl)-2-propanone* [175]

All compounds were dissolved in DMSO

3.6.5. Acridine derivatives: aza mono, bi- and tricyclic compounds

4-[(2'-Diethylaminoethyl)amino]-pyridine (**2**),

4-[(2'-Diethylaminoethyl) methylamino]-pyridine (**3**),

7-Chloro-4-[(2'-diethylaminoethyl)amino]-quinolone (**4**),

7-Chloro-4-[(2'diethylaminoethyl)-methylamino]-quinolone(**5**),

6-Chloro-2-methoxy-9-[(2'-diethylaminoethyl)amino]-acridine (**6**),

6-Chloro-2-methoxy-9-[(2'-diethylaminoethyl)methylamino]acridine (**7**),

2,8,10-Trimethyl-4,6-bis[N-(2'-diethylaminoethyl)-N-methylamino) ethoxy] pyrido-[3,2-g] quinoline (**21**)

(*2,8,10-Trimethyl-bis-4,6-[N-(2'-diethylaminoethyl)-N-methylamino] pyrido-[3,2-g] quinoline* (**24**)

2,8,10-Trimethyl-6-phenoxy-4-[N-(2'-diethylaminoethyl)-N-methylamino] pyrido-[3,2-g] quinoline (**25**) [178]

All compounds were dissolved in DMSO

3.6.6. Benzo[b]-1,8-naphthyridine-5-one derivatives

Benzonaphthyridine-5-one 10-(2'-dimethylaminoethyl) (**1**),

Benzonaphthyridine-5-one 10-(3'-dimethylaminopropyl) (**2**),

Benzonaphthyridine 5-one 10-(2'-diethylaminoethyl) (**3**),

Benzonaphthyridine-5-one 10-(2'-diisopropylaminoethyl) (**4**),

Benzonaphthyridine-5-one 10-(2'-pyrrolidonoethyl) (**5**),

Benzonaphthyridine-5-one 10-(2'-(N-methyl)-pyrrolidinoethyl) (**6**),

Benzonaphthyridine-5-one 10-(2'-piperidinoethyl) (**7**),

Benzonaphthyridine-5-one 10-(3'-piperidinopropyl) (**8**),

Benzonaphthyridine-5-one 10-(2'morpholinoethyl) (**10**).

All derivatives were tested as the hydrochloride salt and dissolved in PBS[179].

4. METHODS

4.1. Determination of minimum inhibitory concentration (MIC)

Dispensing and diluting of bacteria / compounds / reagents to a 96 well microplate were conducted with the aid of an 8 multi barrel pipette. First the half dilution series of the compounds was prepared on the plate in sterile physiological saline. An overnight preculture of the bacterial strain was diluted to 10^{-4} in 2x MTY broth (about 1.0 McF), and 50 µl of this bacteria suspension was added to the each 50 µl of the dilutions of the compounds on the microplate. The microplates were incubated at 37 °C for 24 hours. 10 µl of the solution of MTT (5 mg/ml in phosphate buffered saline (PBS)) was added to each well in order to evaluate the rate of bacterial growth. In this manner, mitochondrial dehydrogenase of living cells reduces MTT resulting in a blue coloured formazan salt. The plates were further incubated for 4 h at 37 °C, and then the MIC values of the compounds against the bacterial strain was determined.

4.2. Checkerboard microplate method for evaluating the combined effect of two drugs [180]

The effect on bacteria of two drugs in combination can also be studied. The half dilution of drug 'A' is made by the above mentioned method. No drug 'A' was put into 12th column (control of drug 'B'). The half dilution of drug 'B' is prepared in selected glasses and 50µl of each dilution was added to each well per row. The concentration of solutions decreases in the direction from up to down. No drug 'B' was added into H row (control of drug ' A'). The bacterial strains were cultured and diluted the same way mentioned above but 100µl of 10^{-4} diluted bacterial suspension was added into each well. The plates were incubated for 24 hours at 37°C. MTT (10 µl) was added to the wells and incubated further 4 hours at 37°C. According to the colour of MTT the further rates can be determined: MIC values of drug 'A' and drug 'B' separately and MIC values of drug 'A' in combination and MIC values of drug 'B' in combination. According to the further rates the effect of drug combination can be determined.

$$FIC_A = MIC_A comb / MIC_A alone \qquad FIC_B = MIC_B comb / MIC_B alone$$

$$FIX = FIC_A + FIC_B$$

0,51 < FIX < 1 → additive effect; FIX < 0,51→ synergism; 1 < FIX < 2 →indifferent effect; FIX > 2→ antagonism

4.3. Transformation of bacteria with pBR 322 plasmid

1 ml of an overnight YTB culture of an ampicillin and tetracycline sensitive bacterial strain was added to 100 ml YTB broth and incubated at 37°C until an optical density (OD) of 0,25-0,30 at 600 nm was reached. The culture was transferred to an ice bath for 10 minutes, centrifuged at 4500 rpm for 10 minutes at 4 °C, then supernatant was removed and pellet re-suspended in ice cold 50 ml 0.1 M $MgSO_4$, centrifuged in cold tube at 4500 rpm for 10 minutes, the supernatant removed and the pellet was re-suspended in ice-cold 3.3 ml 0,1 M $CaCl_2$ and incubated for 1 hour in an ice bath. 200 µl from these cultures containing the competent cells were transferred into tubes containing 1µl of pBR322 (plasmid carrying the genes for ampicillin and tetracycline resistance) and the tubes kept in an ice bath for 30 minutes and then rapidly transferred to 42°C for 1 minute in order that the cells be "shocked". One ml of YTB broth was added to the "shocked" cells and the tubes incubated for 1 hour at 37 °C. These cells were centrifuged in an Eppendorf centrifuge for a few seconds and 800 µl of supernatant removed. The cells were re-suspended in the remaining supernatant and an aliquot of 200 µl was plated onto YTB agar supplemented with tetracycline and ampicillin. Colonies present on these plates were indicative of containing the plasmid that bestowed them with resistance to ampicillin and tetracycline.

4.4. Method for elimination of pBR322 plasmid from *E. coli* AG100 and AG100A strains with replica plating

One colony of bacteria transformed with pBR322 plasmid was added to MTY broth media (5 ml) supplemented with glucose and $MgSO_4$ and incubated for 24 h at 37 °C. From a 10^{-4} dilution of this overnight initial culture 2 ml was transferred to tubes containing 200 ml of MTY broth media, mixed, and distributed in 5 ml aliquots in test tubes. Different concentrations of curing compounds (compounds known to cause the elimination of plasmids) such as promethazine were added to the cells which were then incubated for 24 h at 37°C. 10^4 and 10^5 dilution of these cell suspensions were made and aliquots of 100 µl plated onto YTB agar and the plates incubated for 24 h at 37 °C. The colonies present on these Master plates were transferred by velvet replica plating technique onto YTB agar

containing ampicillin and tetracycline (Replica plate). The plates were further incubated at 37 °C 24 h after which time the distribution of colonies present in each of the respective plates was compared by simple over-laying methods. Colonies present on replica plates contain the pBR 322 plasmid which codes for resistance for ampicillin and tetracycline. Colonies present on the master plate and which do not grow on the Replica plate provide evidence of plasmid elimination promoted by promethazine or any other compound tested. Comparing the number of colonies on both plates provides an estimate of percent plasmid elimination (cure index) produced by a given compound.

4.5. Method for determining antimotility effect of drugs on *P.vulgaris*

From the overnight MTY culture of bacteria 100 µl was added to 900 µl of PBS which contains the drug in subinhibitory (sub MIC) concentration: 10, 90 and 50 percent of the MIC values of certain drugs were administered. PBS with no drug was used as a negative control and promethazine applied in the sub MIC concentration mentioned above was used as a positive control. The samples in Eppendorf tubes were incubated for 15 min at 37 °C. One drop of sample was placed on a microscopic slide and covered with 18 mm square coverslip. The preparation was examined with a phase contrast Zeiss microscope with 63x water objective in the case of *P. vulgaris.* 200-300 cells of *P.vulgaris* were totally counted from 4-6 fields using hand-tally counter. The motile (running), the non-motile and the vibrating (tumbling) cells were counted separately. The ratio of cells with different moving was expressed in percentage of untreated control.

4.6. Multidrug resistance reversal effect by decreasing Rhodamine 123 efflux

The L51478 mouse T cell lymphoma cell line was infected with the *mdr1/A* retrovirus. *mdr1* expressing cell lines were selected by culturing the infected cells with 60 ng/ml colchicine to maintaine expression of the mdr phenotype. The L5178 MDR cell line , and the L5178Y PAR cell line were grown McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine (1 ml/100ml), streptomycin (1 ml/100ml) and nystatin (0.1 ml/100ml). The cells were adjusted to a concentration of $2x10^6$ / ml and resuspended in serum free McCoy's 5A medium and the cells were distributed into 0.5 ml aliquots to Eppendorf centrifuge tubes. Then the tested compounds were added in various concentrations of the 1.0 mg/ml stock solutions, and the samples were incubated for 10 min at room temperature. Then 10 µl (5.2 µM final concentration) indicator R123 was added to

the samples, and the cells were incubated for a further 20 min at 37 °C, washed twice and resuspended in 0.5 ml PBS for analysis. The fluorescence of cell population was measured by flow cytometry with a Beckton Dickinson FACScan instrument. Verapamil was used as a positive control in the R123 exclusion experiments. The percentage of the control mean fluorescence intensity was calculated for PAR and MDR cell lines as compared to untreated cells. An activity ratio was calculated by the following equation on the basis of measured fluorescence values:

$$FAR = (MDR_{treated} / MDR_{control}) / (PAR_{treated} / PAR_{control})$$

5. RESULTS

5.1. Experiments on bacteria having proton pump transporter with resistance modifier

5.1.1.The interaction of Ca-channel blockers (promethazine, (±)verapamil) and potential proton pump inhibitor trifluoro-methylketones on *E. coli* AG100 and AG100A strains

In preliminary experiments, the interaction of promethazine of potent antimicrobial effect and newly synthesised trifluoromethyl-ketones was evaluated on both strains respectively in agar diffusion method to use sterile filter paper strips containing 50 μg promethazine and 200 μg TF compounds. The strips containing promethazine and a representative of TF compounds were put at right angles to each other on the MTY agar containing the bacterial culture. After incubation the type of interaction was evaluated. TF 50, 19, 20 were ineffective, TF 5 had very slight , TF 10 and 11 had strong and TF 18 had very strong synergistic effect with promethazine on both strain (data not shown). TF 11 had slight antibacterial effect on both strain, TF 10 exerted stronger inhibition only on mutant strain however TF 18 inhibited the growth of both strain strongly.

For the further studies of interaction the MIC values of promethazine and TF compounds were determined in broth dilution method (Table 1.)

Table 1. The MIC-values of Ca-antagonists, TF compounds and antibiotics on *E. coli* AG100 and AG 100A strains

Compounds	**MIC values (μg/ml)**	
	E. coli AG 100 (wild)	*E. coli* AG 100 A (mutant)
promethazine	156.25	78.15
verapamil	500	250
TF18	7.8	3.9
TF10	31.25	7.8
TF50	1250	625
TF5	1250	39.1
TF51	313	156
TF20	1250	1250
TF19	1250	312.5
TF11	62,5	31,25
TF6	1250	625
ampicillin	5	1.25
teracycline	40	10
erythromycin	80	1.25

Based on the preliminary experiments the three most effective TF compounds (TF 10, 11 and 18) were examined in checkerboard experiments with promethazine. After determining the MIC values of compounds employing with or without combination, the FIX index was calculated which is characteristically for the phenomenon of the interaction. Data are shown on table 2.

Table 2. The interaction of promethazine with TF 10, 11, 18 and the combined effect of verapamil and TF 18 on *E. coli* AG100 and AG 100A strains

Compounds	**MIC (µg/ml) in**	
	***E. coli* AG 100**	***E. coli* AG 100 A**
Promethazine	156,25	78,15
TF 10	31,25	7,8
Promethazine + TF 10	78,15 + 7,8	39,05 + 3,9
FIX index / interaction	0,75 / ADDITIVE	1,0 / ADDITIVE
TF 11	62,5	31,25
Promethazine + TF 11	39,05 + 15,65	19,55 + 7,8
FIX index / interaction	0,5 / ADDITIVE	0,5 / ADDITIVE
TF 18	7,8	3,9
Promethazine + TF 18	39,05 + 1,0	19,55 + 1,0
FIX index / interaction	0,38 / SYNERGY	0,51 / ADDITIVE
Verapamil	500	250
Verapamil + TF 18	125 + 15,63	125 + 1,95
FIX index / interaction	2,25 / ANTAGONISM	1,0 / ADDITIVE

According to the checkerboard technique synergistic effect between TF compounds and promethazine was exhibited only by the combination of TF 18 with the Ca-calmodulin channel blocker promethazine just on the proton pump expressing *E. coli* strain. TF 10, 11 showed moderate combined effect on both *E. coli* strain. According to the experiments the most effective compound (TF 18) was examined further by the combination with the Ca-channel blocker (±)verapamil on both *E. coli* strain. Interestingly on the mutant strain the combination was additive however on the proton pump expressing wild strain an antagonism was demonstrated.

5.1.2. Elimination of pBR 322 plasmid from *E. coli* AG100 and AG100A strains by promethazine

The calmodulin inhibitory promethazine is long known to have antiplasmid effect [164]. We were wonder whether the existence of proton pump in the bacterial membrane can influence this phenomenon. The plasmid curing ability of promethazine was evaluated with

the use of the two *E. coli* strains mentioned above transfected with pBR322 plasmid (tet^r, amp^r). As shown by Table 3, promethazine promotes the elimination of plasmids from the wild type *E. coli* strain in a concentration dependent manner such that the maximum curing effect takes place at a concentration just below the MIC of the compound (120 mg/l). In contrast to the wild type strain, promethazine causes elimination of plasmids from the proton pump-deleted mutant at lower concentration of the compound (80 vs. 140 mg/l).

Table3. Elimination of pBR 322 plasmid with promethazine from *E. coli* AG100 (wild) and AG100A (mutant) strains.

	***E.coli* AG 100 (wild)**	***E.coli* AG 100 A (mut)**
promethazine (μg/ml)	Colonies without plasmid (%)	
0	0	0
40	0	0
60	0	0
80	0	11,0
100	1,0	MIC
120	13,0	
140	10,0	
160	MIC	

5.1.3.The interaction of potential proton pump inhibitor TF 18 and some representatives of antibiotic groups on *E. coli* AG100 and AG100A strains

The further step was to study whether TF 18 can influence the antibiotic sensitivity of both *E. coli* strain. It is well known, that the AcrAB-TolC proton efflux pump has its antibiotic substrate of large hydrophobic characteristic e.g. erythromycin and tetracycline. It is proved on the Table 4., which shows the MIC values of antibiotics and TF18, in proton-pump-deleted and –expressing strain as well. Hydrophilic ampicillin was also employed as a control. According to the results of the checkerboard technique TF 18 at a concentration well below its MIC and in combination of tetracycline, erythromycin each of which is also well below its MIC, have a synergistic effect on the inhibition of both strain, however the lower FIX index in the case of proton-pump-expressing wild strain indicates the stronger synergism than that of in the case of proton-pump-deleted mutant strain. Remarkable that the MIC value of erythromycin which is proved to be the substrate of AcrAB-TolC proton efflux pump [84] decreased 32 fold in the presence of TF 18, in the proton-pump-expressing strain (from 80 mg/l to 2,5 mg/l), however this decreasing was only 4 fold in the mutant strain.

Expectedly the MIC values of ampicillin did not change significantly in combination with TF 18 in both strain.

Table 4. The interaction of TF 18 and some representatives (ampicillin, tetracycline, erythromycin) of antibiotic groups on *E. coli* AG100 and AG100A strains

Compounds	***E. coli* AG100**		***E. coli* AG100A**	
	MIC values (mg/l)	FIX values (type of interaction)	MIC values (mg/l)	FIX values (type of interaction)
TF18	7.8		3.9	
AMP	5		1.25	
AMP+TF18	2.5 + 3.9	1.0 (additive)	1.25 + 3.9	2.0 (indifferent)
TET	40		10	
TET+TF18	2.5 + 1.95	0.31 (synergy)	1.25 + 0.98	0.38 (synergy)
ER	80		1.25	
ER+TF18	2.5 + 1.95	0.28 (synergy)	0.3 + 0.5	0.38 (synergy)

5.1.4. Comparison of the interaction of erythromycin plus known proton pump inhibitor Omeprasol with that of erythromycin plus the newly synthesised TF 18 – a potential proton pump inhibitor-and with its structurally close derivative TF 51 on *E. coli* AG100 and AG100A strains.

To determine the specificity of proton pump inhibitory effect, a known and two newly synthesised TF compounds were compared in the combination experiment with erythromycin on both *E. coli* strain. Data are shown on Table 5.

Table 5. Comparison of proton-pump inhibitor Omeprasol with TF 18 in combination with erythromycin on *E. coli* AG100 and AG100A

Compounds	***E. coli* AG100**		***E. coli* AG100A**	
	MIC values (mg/l)	FIX values (type of interaction)	MIC values (mg/l)	FIX values (type of interaction)
Erythromycin	80		1,25	
TF 18	7,8		3,9	
TF 51	313		156	
Omeprasol	2500		2500	
TF 18 + ER	1,95 + 2,5	0.28 (synergy)	0,5 + 0,2	0.29 (synergy)
TF 51 + ER	157 + 40	1.0 (additive)	156 + 3,9	2.0 (indifferent)
OMP + ER	1250 + 40	1.0 (additive)	2500 + 3,9	2.0 (indifferent)

As seen in the table 5. Omeprasol can influence the erythromycin sensitivity nor in the case of *E. coli* AG100, neither in proton pump-deleted mutant. A very marginal inhibitory effect (additive effect, FIX-index= 1.0) takes place in *E. coli* operating with proton efflux pump.

Exactly the same what is to explain about TF 51, however the structural differences between TF 51 and 18 are only a terminal H_3 and F_3 group in the side chain.

5.1.5. Combinations of antibiotics (erythromycin and ampicillin) with known resistance modifiers (promethazine, verapamil) respectively on *E. coli* AG100 and AG100A strains

After a newly synthesised potent resistance modifier trifluoro-methylketones (TF18) was found to be successful in reversing antibiotic resistance caused by a proton efflux pump, known resistance modifiers, the Ca-calmodulin-antagonists promethazine, and the Ca-antagonists verapamil, were employed in combination with erythromycin (substrate of AcrAB efflux pump due to its hydrophobic characteristic) and with ampicillin (surely not substrate of the Acr AB efflux pump due to its hydrophilic phenomenon) in the proton efflux pump-deleted and in the wild *E. coli* strains. Table 6 shows the results deriving from the checkerboard technique.

Table 6. Combined effect of ampicillin, erythromycin with Ca-antagonists promethazine, verapamil on *E. coli* AG100 and AG100A strains.

Compounds	***E. coli* AG100**		***E. coli* AG100A**	
	MIC values (mg/l)	FIX values (type of interaction)	MIC values (mg/l)	FIX values (type of interaction)
Erythromycin	80		1,25	
Ampicillin	5,0		1,25	
Promethazine	156,25		78,15	
Verapamil	500		250	
Ery + PMZ	20 + 78,13	0,75 / ADDITIVE	0,33 + 19,53	0,5 / ADDITIVE
Ery + VP	80 + 500	2 / INDIFFERENT	0,33 + 15,7	0,32 / SYNERG.
Amp + PMZ	5,0+156,25	2 /INDIFFERENT	2,5 + 78,13	3 / ANTAGON.
Amp + VP	2,52 + 250	1 / ADDITIVE	2,5 + 250	3 / ANTAGON.

In the wild type *E. coli* AG100 strain promethazine only slightly (in additive way), verapamil did not influence the sensitivity of erythromycin which is substrate of the pump of the bacteria. In the proton pump deficient bacteria the Ca-calmodulin antagonist promethazine caused a strong additive effect but not synergy, however the Ca-antagonist verapamil decreased the MIC of erythromycin to 0,33 mg/l like promethazine did, but promethazine needed its 25% of MIC value (19,53 mg/ l) for this interaction, however verapamil was able to reach the same only with its 5 % of MIC value (15,7 mg/l), namely the verapamil + erythromycin interaction is synergistic in the proton-pump deficient strain.

The sensitivity of ampicillin wasn't influenced by both Ca-antagonists on wild strain, however interestingly promethazine and verapamil stood in antagonism with ampicillin on the mutant strain.

5.1.6. Inhibition of motility by trifluoro-methylketones on a clinical isolates of *P. vulgaris*

The movement of bacterial flagellae is strongly energized by proton motive forces, so the fuel for movement comes from the energy of proton gradient (details in discussion). This fact was used to study TF compounds further, whether really having an effect on proton pumps and so being able to inhibit the movement of bacteria. Two types of movement were examined: the running and the tumbling and of course the non-motile cells. Since the drugs were used in subinhibitory concentrations, first the MIC values of TF compounds were determined (table 7.). Ren et al suggested that phenotiazines e.g. promethazine can inhibit the movement of bacteria by reversible inhibition on the proton pump of the bacterium. Using this hypothesis promethazine was used as a positive control also in subinhibitory concentration.

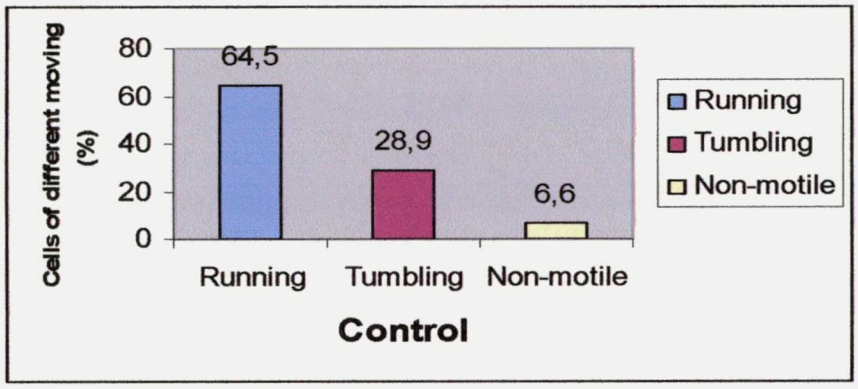
Table 7. MIC values of TF compounds and promethazine on *P. vulgaris*

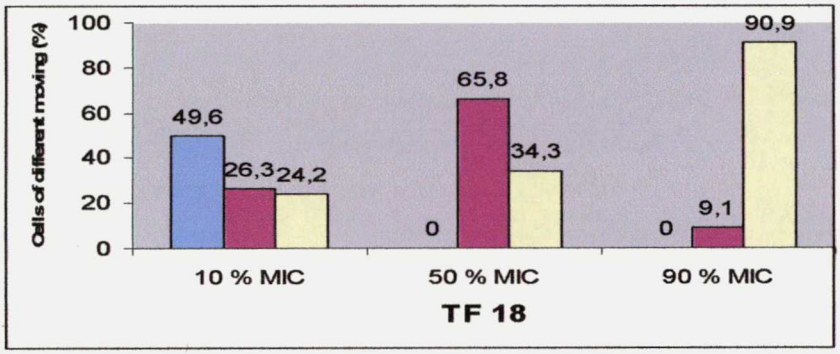
Compounds	MIC values (mg/l)
TF 18	6,3
TF 10	12,5
TF 11	156,2
TF 6	625
TF 5	625
TF 51	625
TF 20	1250
TF 19	1250
promethazine	156

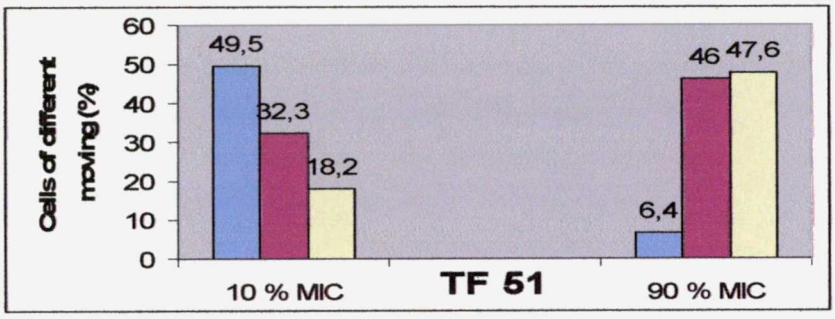
In preliminary experiments the strain of *P. vulgaris* was incubated with 10, 50 and 90 % of MIC of TF 18 for 15 min at 37 °C respectively, then the suspension was plated on an agar plate. No drug was used as a negative control. Counting the number of colonies grown, there were not significant differences between the treated and untreated samples, indicating that the treatment with the sub-MIC concentration of TF 18, in a short period, does not influence the growth of bacteria.

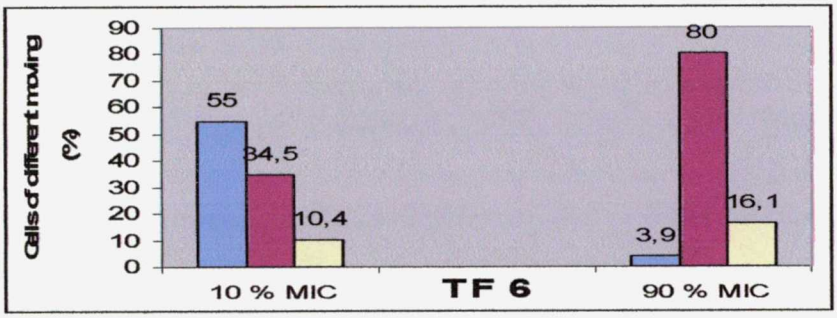
In the antimotility experiment the average distribution of untreated *Proteus* movement was the following: 64.5± 10 % running, 28.9±8 % tumbling and 6.6± 3 % non-motile, respectively. Compounds **5, 19, 20** at 10 % of their MIC partially inhibited the motility: the

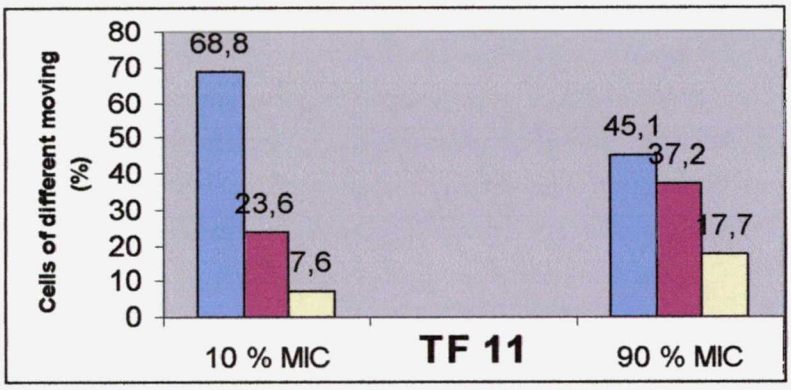
ratio of running cells decreased, however one of the tumbling cells significantly increased. The number of non-motile cells increased somewhat only in the sample treated with **TF5** at 10 % of MIC. However **TF 5, 19, 20** had solubility problems and formed slight opalescence which could inhibit the bacterial motility. **TF 6, 10, 11, 18 and 51** could exert real inhibition of moving: the ratio of non-moving cells treated with these compounds increased significantly. In the case of **TF 6** and **18** the number of tumbling cells also changed however **TF10, 11** and **51** didn't alter them significantly. The correlation between concentrations applied and antimotility effect was significant in the case of **TF 6, 18** and **51**. Less correlation was observed with **TF 11** and no correlation could be established with **TF 10**. **TF 18** exerted the most remarkable inhibition of movement: the ratio of non-moving cells increased significantly in a concentration- dependent manner. Already at 50% of its MIC no cells were running and at 90 % of its MIC 90,9 % of bacteria were totally non-motile and only 9,1 % of the cells showed tumbling without running. As it was expectable among eight TF compounds **TF 18** was the most promising drug, with a strong antimotility effect on *P. vulgaris* (Figure 7.) reinforcing its proton pump inhibitory action supposed in earlier interaction studies.

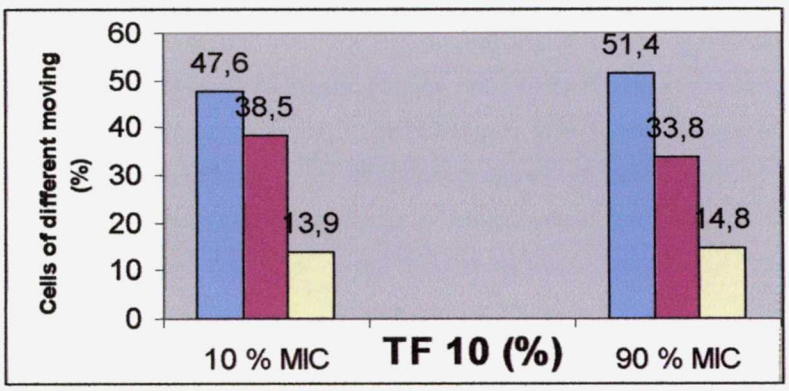





Cells of different moving (%)
60
50
40
30
20
10
0
49,5
32,3
18,2
6,4
46
47,6
10 % MIC
TF 51
90 % MIC


Cells of different moving (%)
90
80
70
60
50
40
30
20
10
0
55
34,5
10,4
3,9
80
16,1
10 % MIC
TF 6
90 % MIC


Cells of different moving (%)
80
70
60
50
40
30
20
10
0
68,8
23,6
7,6
45,1
37,2
17,7
10 % MIC
TF 11
90 % MIC


Cells of different moving (%)
60
50
40
30
20
10
0
47,6
38,5
13,9
51,4
33,8
14,8
10 % MIC
TF 10 (%)
90 % MIC

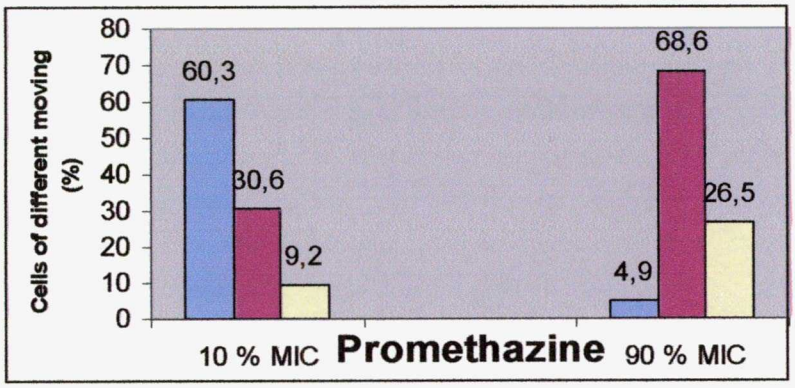


Figure 7. The antimotility effect of TF compounds and the positive control promethazine on *P. vulgaris*

5.2. Experiments with tumour cells having ABC transporter and resistance modifier agents

5.2.1. Reversal of multidrug resistance in mouse lymphoma cells by mono, bi and tricyclic acridine derivatives

Acridine compounds and other tricyclic derivatives have already been tested and several derivatives have interesting anti MDR activity (Hever et al). The aim of these examinations was to clarify the functional groups, which actually interact with the P-gp 170 protein. Several variations have been made concerning: 1. the aromatic moiety e.g. pyridine (**2**,**3**), quinoline (**4**,**5**), acridine (**6**,7) and pyridoquinoline (**21, 24, 25**) derivatives have been investigated, 2. the side chain has been modified e.g. X atom nature (O, N or S) and / or size of the side chain (see chemical structure in annex, chapter 2 and 3.).

In the Rhodamine 123 accumulation assay on the L5178 mouse lymphoma cell line the follow were found (Figure 8.): the two examined pyridine derivatives namely **2** and **3** are inactive. Quinolines **4** and **5** are as active as positive control verapamil. The amino-substituted compound, **5** has a little better activity than the non substituted **4** at 2 mg/l concentration. High activity is observed for tricyclic derivatives. Acridine derivatives **6** and 7 present a similar activity at low concentration but the non-substituted compound, **6** could be toxic at high doses. Among pyridoquinolines **24, 25** exerted the best activity.

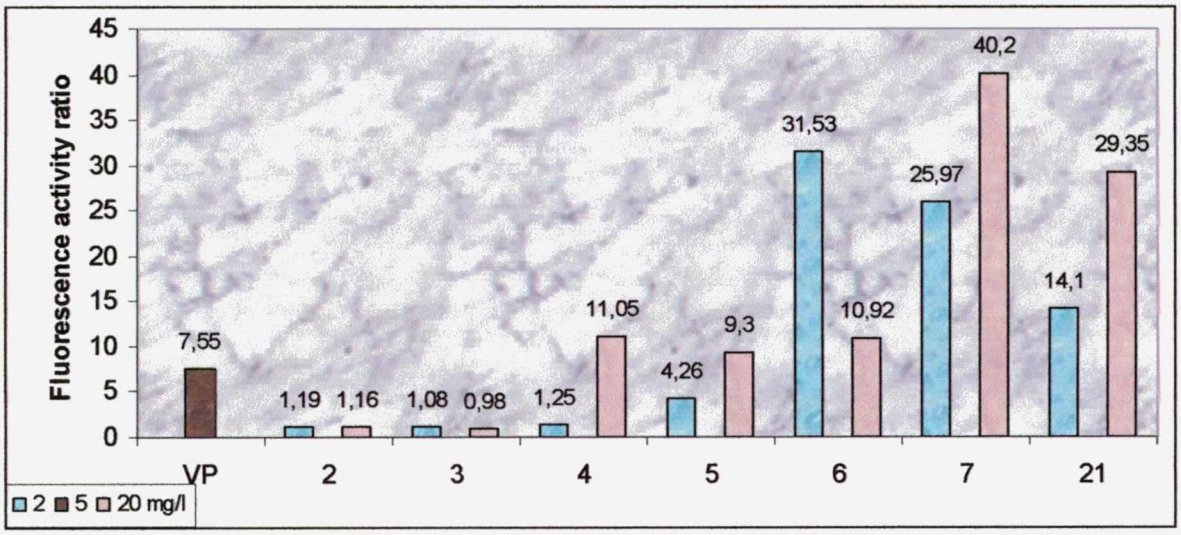


Figure 8. MDR reversal effect of tricyclic compounds, where **2**, **3** are aminopyridine, **4**, **5** are aminoquinoline, **6**, **7**, **21** are aminoacridine derivatives.

The most active compounds –**6**, 7, **21, 24** and **25** were examined at 0,2 and 2 mg/l concentration as well.

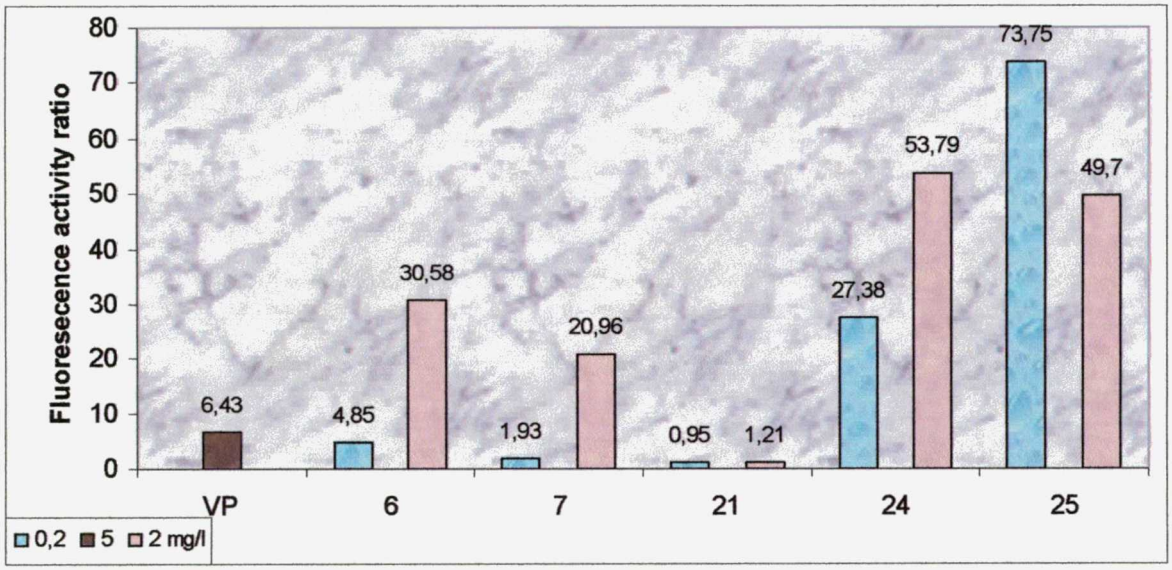


Figure 9. MDR reversal effect of the most active tricyclic compounds at lower concentrations

As seen on Figure 9. **6**, 7 and **21** had no anti MDR activity at 0,2 mg/l concentration, however **24** and **25** exerted the strongest anti MDR effect among tricyclic derivatives even at 0,2 mg/l concentration. The lower fluorescence activity ratio values in higher doses can be due to potential toxicity.

5.2.2. Reversal of multidrug resistance in mouse lymphoma cells by benzo [b]-1,8-naphthyridine derivatives

Because of its structural similarity with acridines, benzonaphthyridine nucleus was selected to study the efflux pump inhibitory effect.

Among compounds tested **1** had very weak anti MDR effect at both concentrations.**2**, **4**, **7** exerted strong reversing activity only at the higher 20 mg/l concentration.**5**, **6** and **8** showed the most promising MDR-reversing activity at lower concentration as well in comparison with reference drug verapamil. **5** being the most active of the set and was probably toxic in higher doses. It is important that all of these derivatives (namely **5**, **6** and **8**) are substituted with an azaheterocyclic group (Figure 10.).

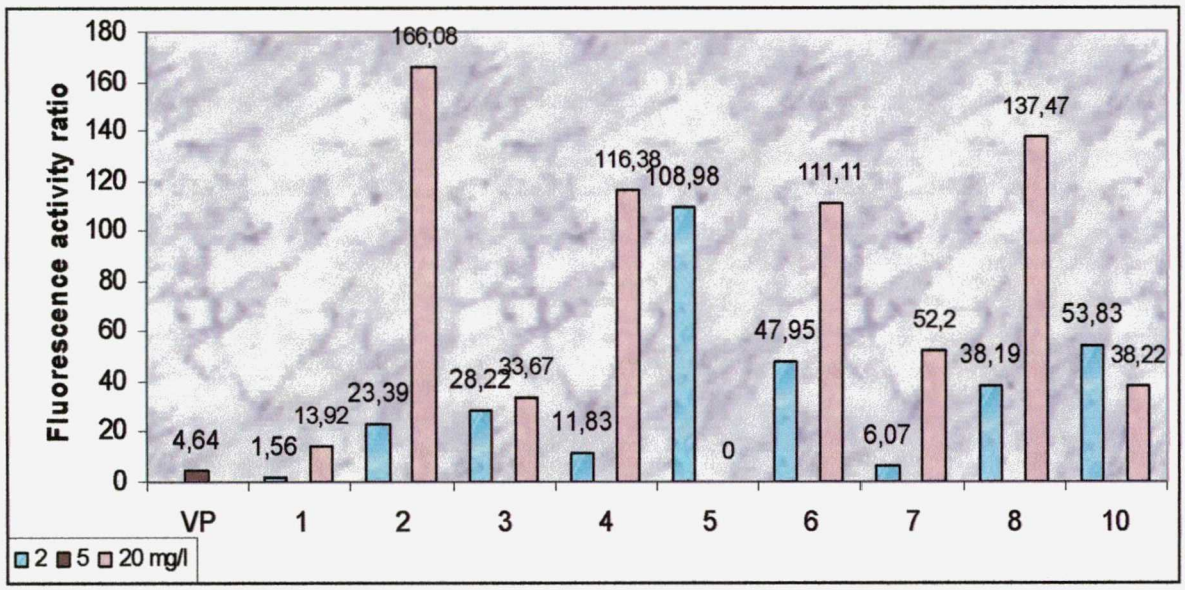


Figure 10. MDR reversal effect of benzonaphthyridine-5-one derivatives, where **5** was toxic at 20 mg/l concentration

6. DISCUSSION

6.1. Experiments on bacteria having proton pump transporter with resistance modifier agents

In the clinical practice with the more and more frequently appearing multiresistant bacterial strains, causing lethal infections due to therapeutic failure, there has been urging need to solve this world-wide problem. The solution can be the introduction of new antibiotics, but the inhibition of resistance mechanisms by known and newly synthesised resistance modifier agents might be cheaper and more effective.

Earlier it was found, that well-known psychotropic drugs, e.g. phenotiazines have antibacterial and antiplasmid effect [162,164,166,168,170,181].

Since the elimination of plasmids never occurs in the whole bacterial population, a more effective mechanism was chosen for reversing drug resistance: the inhibition of drug efflux pumps by chemical agents.

6.1.2. Combined effect of known and newly synthesized resistance reversing agent and some representatives of antibiotics on *E. coli* AG100 and AG100A strains. The correlation between chemical structure of TF compounds, and their antibiotic resistance reversing effect.

The newly synthesised trifluoro-methylketones have already been shown to have a strong inhibitory effect against *Helicobacter pylori* compared with clinically used metronidazole and clarithromycin [182]. Taking further this evidence we examined whether they have antibacterial effect on other bacteria and their resistance reversal ability by inhibition of proton efflux pump was also examined.

Among the nine TF compounds, TF **18** (1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone) derivative exerted the strongest antibacterial activity on the proton pump-bearing and – mutant *E. coli* strains as well. Since TF**10**, **11** and **51** derivatives have also quite low MIC values on both strain, these four compounds were examined further in combination experiments. Only TF **18** showed some effect on the wild type *E. coli* strains combined with known resistance modifier promethazine and verapamil respectively, namely it potentiated the antibacterial action of promethazine indicating that this phenothiazine can be a target of the efflux pump, however with verapamil the combination was an antagonism suggesting, that TF **18** probably makes difference between the calmodulin-antagonistic and Ca-channel blocker property and the proton pump has some role on these effects [183].

After transformation of the two *E. coli* strains by pBR322 plasmid, practically two strains were observed considered clinical resistant strain. Elimination of the plasmid by promethazine from both strain showed that promethazine could be the substrate of the AcrAB efflux pump, since the maximal elimination rate was significantly higher in wild type than mutant one, and that's why the presence of this efflux pump could influence the plasmid elimination with promethazine. Later it was shown that the plasmid curing effects of promethazine, trifluoperazine and 9-aminoacridine were increased in the presence of a trifluoroketone proton pump inhibitor, TF **18**, on *E. coli* K12 LE140 strain in a model experiment, where compound TF **18** alone didn't exert antiplasmid effect [184]. It is proposed that the inefficient penetration of antiplasmid compounds could be responsible for the weak plasmid-curing effect in some clinical isolates, and that membrane active, calmodulin- and proton pump inhibitors may be combined for plasmid curing in antibiotic-resistant bacteria.

Considering the evidence, that the erythromycin is the substrate of AcrAB efflux pump due to its large hydrophobic characteristics [84], this antibiotics and the similar tetracyclin were combined with TF **18** on both *E. coli* strain. The hydrophilic ampicillin (with this phenomenon not substrate of AcrAB pump) was also employed as a control. TF **18** decreased the MIC value of erythromycin 32 fold in wild strain (FIX index= 0,28 / strong synergy), and only 4 fold in mutant strain (FIX index= 0,38 / slighter synergy). Tetracycline and TF 18 also showed a synergism, however -as it was expected- the MIC value of ampicillin wasn't influenced by TF 18 significantly. These results indicate that TF 18 really can have a special effect on inhibiting this proton efflux pump. The slighter synergy of TF 18 and erythromycin on proton pump-deleted mutant strain can be explained only by the fact that TF 18 itself also has a strong antibacterial effect. As far as the ampicillin is considered, the differences between the two strains in MIC values cannot be explained directly by the presence / existence of proton efflux pump, all the more by the membrane permeability differences which is due to the efflux pump.

Comparison of the effect of TF **18** with other proton pump inhibitors e.g. omeprasol and the structurally related TF **51** it was observed, that omeprasol and TF **51** had only additive effect with erythromycin on the wild strains (FIX index=1,0 / additive effect) indicating having no or very slight proton pump inhibitory effect on the AcrAB efflux system. However the structural differences between TF **18** and TF **51** are only a terminal F_3 and H_3 groups in the side chains of the benzoxazole ring. Considering these results an evidence is suggested that

the AcrAB efflux pump needs a certain structural requirement in the group of proton pump inhibitors for its successful inhibition.

Finally two known resistance modifier agents were examined in combination with the former antibiotics on the two *E. coli* strains. Neither of them could exert similar decreasing in MIC values of erythromycin on proton pump positive strain what TF **18** could. Interestingly the Ca-channel blocker verapamil was synergistic with erythromycin on the proton pump-deficient strain. This phenomenon can be explained by a changing effect of verapamil in the membrane permeability, which cannot be specific for its Ca-antagonistic property, since the Ca-calmodulin antagonistic promethazine did not show similar effect (additive with erythromycin on mutant strain). Interestingly the ampicillin with both Ca-antagonists stood in antagonism in mutant strain namely the Ca-channel blockers somehow could decrease the membrane permeability. Moreover the effect of verapamil on mutant strain can be in correlation with the properties of the antibiotics combined with. With hydrophobic erythromycin synergism, with hydrophilic ampicillin antagonism effect was found indicating the difference membrane permeability requirements of the two different type of antibiotics.

Comparing first the chemical structure of the trifluoro-methylketones examined for antibacterial effect it is suggested that the replacement of the benzoxazol residue of the most potent TF**18** with other aromatic or heteroaromatic rings with intact side chain (resulting TF**19,20**) caused strong decrease in antibacterial action indicating that the benzazole derivatives were more active than either benzene or azole derivatives [183]. However after the TF **18** the most potent TF compound is TF **10** having only a benzole ring instead of benzoxazol, but its side chain bears an additional carbonyl group directly next to the ring which could mean the antibacterial efficacy. However next to intact rings the side chain could have also role in antibacterial effect, since removal of the terminal F_3 from TF **18** to H_3 resulting TF**51** causes approximately 40 fold increasing in MIC values of both *E. coli* strain. Removal the methylene from the side chain of TF **18** resulting TF**6** directs to 160 fold increasing in MIC of both strain. Summarized the structure of the benzoxazole ring and the side chain found in TF**18** are together similarly important for the antibacterial action of trifluoro-methylketones. In combination experiments the three TF compounds (TF**10,11,51**) next to TF **18** were applied having the lowest MIC values. Considering the results obtained, it is sure that the side chain with its terminal F_3 group is essential for proton pump inhibition but the benzoxazol ring has to be also some role in drug reversary action, since TF**11** wasn't effective in combination experiments. Further evidences were obtained between chemical

structure of trifluoro-methylketones and their proton pump inhibitory effect in antimotility experiments.

6.1.3. Inhibition of motility on *P. vulgaris*

For reinforcing the finding, that some TF compounds exert their resistance modifier effect by inhibition of proton pump, the antimotility effect of these drugs was studied on a clinical isolates of a *P. vulgaris* strain.

It is well known that the fuel for rotation of bacterial flagellae is the membrane gradient of protons [185,186]. The flagellar motor can rotate counterclockwise (CCW), where the several filaments on a cell join in a bundle and drive the cell forward resulting the running movement. In the case of clockwise (CW) rotation of flagellae the filaments bundle are disrupted causing a somersaulting movement or tumbling. Cells can direct their movement by regulating switching between CW and CCW rotation [187,188]. The torque for flagellar rotation is generated at the base [189,190]. The basal body functioning as a motor contains the essential proteins for moving: the so-called non-rotating stator complex comprises Mot A and Mot B membrane proteins which function together to conduct ions (protons) across the membrane [191-196]. The rotating part of basal body is the rotor or switch complex [197]. It contains the Fli M protein having main role in CW/CCW switching [198], the Fli G main rotor protein with any additional interacting, role mainly with the stator Mot A [199,200,201], and Fli N with structural role [202]. The motors can convert chemical energy into mechanical energy: as rotation is driven by cyclic conformational changes in the stator, which occurs as protons bind to and dissociate from a critical and essential Asp residue of Mot B. So this residue is essential for rotation and involved directly in proton transfer. The conformational changes would regulate access to the Asp 32 site to ensure that protons entered the site from periplasm and departed to the cytosol. The conformational changes in the stator would apply forces to the rotor, most likely on Fli G [203].

After the explanation the role of proton motive forces in the motility of bacteria, the supposed proton pump inhibitor TF compounds were tested whether having antimotility effect. With this phenomenon the reversal of antibiotic resistance could be attributed to proton pump inhibitory effect.

Earlier it was shown that the known resistance modifier promethazine has antimotility effect [204,205]. After studying the newly synthesized TF compounds with possible resistance reversary effect, some connections were established. In the case of badly soluble compounds (TF **5**, **19**, **20**) the strong decreasing in the number of motile bacteria can only possible due

to the increased viscosity of media because of solubility problems as the rotation speed varies inversely with viscosity of the medium [206]. Among TF compounds having real antimotility effect of different efficacy only TF **18** and **51** showed significant antimotile effect at 10 % of MIC. At 90 % of MIC also TF **18** and **51** were the most effective in concentration-dependent manner as the ratio of non-motile cells were 90,9 % and 47,6 % respectively (in the case of promethazine 26,5 %). TF **6** worked also in concentration-dependent manner however only the number of tumbling cells increased significantly with decreasing of running ones. Taking as a basic of molecular structure of most effective TF **18**, it is suggested that the benzoxazol ring is essential for antimotile effect as ring substitution next to intact side chain strongly decreased this efficacy. Among molecules having the original ring complex (TF **6**, TF **51**) with substituted side chains antimotility effect was more expressed. However in the side chain when terminal trifluorid was substituted with H_3 group (resulting TF 51) antimotility effect was weaker. Probably the terminal trifluorid with benzoxazol ring is essential for strong antimotility effect and maybe for the proton pump inhibition as well. When the molecule of TF **18** was modified by extraction of the methylene group from the side chain (resulting TF **6**), only the number of tumbling cells increased, the number of non-motile cells didn't change significantly. It indicates that this structure may have a role in intervention into the CCW/CW switching and the methyl group may be important for the effective proton pump inhibition. Interestingly in the case of TF **18** with the increasing of applied concentration first the running cells decreased with increasing number of tumbling ones next to no significant alteration of non-motile ones and with further increasing of drug concentration practically the tumbling cells became non-moving. Summarized a progressivity is phenomenal for the antimotility effect of TF**18**: first the running cells become tumbling and later, with increasing of drug concentration, the tumbling ones become non-motile. It is evidence that the inhibition of motility can be by proton pump inhibition and / or decreasing proton motive forces. However it is not closed that the CCW / CW switching and its influence comprises operation of proton pumps / proton motive forces. Taking these suggestions TF **18** can be considered a drug having real proton pump inhibitory effect reinforcing the mechanisms by which TF **18** works as an antibiotic resistance reversing agents.

Other importance of the finding is the inhibition of motility – as a potent virulence factor – itself. It was previously reported that TF **18** inhibited *H. pylori* growth *in vitro* [207]. *H. pylori* is a strongly motile bacterium [208], and the motility conferred by the flagella is necessary for colonization in the gastric mucosa and development of gastritis by *H. pylori*

[209,210]. Therefore, in the stomach there are some connections between the pathogenecity and the active moving of *H. pylori*. Recent studies have provided evidence that motility in *H. pylori* was also suppressed by the proton pump inhibitors such as rabeprazole [209,210]. In this study, it is suggested that TF compounds may have an inhibitory effect against the *in vitro* motility of *H. pylori*. The inhibition of bacterial motility can be related with the virulence of bacteria: the pathogenicity can be decreased in the presence of drugs examined. In the case of nephropathogenic *E. coli* strains the active moving has a role in wandering of bacteria from the lower urine tract, bladder to upper tract via urether to cause chronic pyelonephritis [211]. The adhesive pili have also role in sticking of bacteria. It was proved in electromicroscopic experiments that the function of pili can be inhibited by specific compounds: pili-specific phages failed to adsorbate [212].

Among Gram-negative bacteria the intrinsic resistance for some typically „Gram-positive antibiotics" e.g. erythromycin and for some unrelated drugs, is due to the working of an efflux pump system which uses proton gradient for its operation and work in synergy with outer membrane barrier. The successful inhibition of this proton pump by 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF **18**) means the possibility to employ an earlier ineffective antibiotics with a pump inhibitor in combination. The experiments show, that a so-called „Gram-negative resistance" can be overcome by this method.

6.2. Experiments with tumour cells having ABC transporter and with resistance modifier agents

In the clinical practise to avoid the selection of resistant tumour cells combined chemotherapy has to be employed. Theoretically the co-administration of cytostatic drugs with the proper resistant modifier agent could be also a possible and cheaper way to overcoming multidrug resistance and the therapeutic failure.

A plenty of structurally unrelated resistance modifier drugs were found which can inhibit drug efflux pumps of tumour cells. 3,5-diacetyl-1,4-dihydropyridines were effective to reverse multidrug resistance in mouse lymphoma cell line operating with the P-glycoprotein-170 (P-gp 170) efflux pump protein [213]. On the same laboratory cell line this efflux pump was successfully inhibited by heterocyclic compounds e.g. phenotiazines [214], thioacridine derivatives [215], anti-psychotic drugs [216]. Among plant derivatives some carotenoids [217], diterpenes and triterpenes [218] were also shown to inhibit MDR efflux proteins in mouse lymphoma and human breast cancer cell lines as well. Several plant extracts derived from 'Anastasia Red' sweet pepper fruits [219], from Persimmon *Diospyros*

kaki peels [220], and *Allium victorialis* total herbs [221] exerted strong anti-MDR activity on mouse lymphoma cell line. Feijoa peel extracts had only marginal effects [222]. Since these extracts are chemically not entirely characterized, further additional analysis are needed for finding the structure-activity relationship.

In general the chemosensitizers that block P-gp drug extrusion are lipid-soluble at physiological pH, possess a basic nitrogen atom and at least two co-planar rings [127], have aromatic moieties, but the presence of electron donor groups is a more general feature [223].

6.2.1. Reversal of multidrug resistance in mouse lymphoma cells by mono, bi and tricyclic acridine derivatives

To summarize the results, it was shown that anti MDR activity depends on the aromatic moiety, the bonded X heteroatom and nature of the side chain. A better activity is found: when the aromatic moiety is acridine or pyridoquinoline compared with quinoline and pyridine. It can be questioned whether log *P* (or log *D*) are related to the activity. If the values for pyridine compounds are low (<2) compared with all other products, the calculated values for quinoline compounds **4** and **5** (3,32 and 3,70) are in the same range of an active pyridoquinoline and even higher than that of an other, nevertheless very active pyridoquinoline [178]. The lack of direct correlation between log *P* and anti MDR activity has previously been underlined [224]. If we consider the compound activity related to the number and length of the side chain -keeping the same X atom on the ring- activities are in the following increasing order: when X=O **15**[178]< **21**, when X=N **20**[178] < **24** < **25**, that is 2 side 'short' chains<one side 'short' chain or two side 'long' chains (see Annex chapter 3.). For compound **21** a better activity ensues since its side chain is longer with an additional electron donor group (diethylaminoethyl ring) compared with the other disubstituted pyridoquinolines 15 [178].

Considering more than 100 compounds already tested [225], activities have been correlated with the presence of two or three hydrogen bond acceptor (HBA) groups with a spatial separation of 2,5 or 4,6 Å. For the products studied here, possible HBA groups are tertiary amino groups, heterocyclic nitrogen. When X= N (see Annex chapter 3.) e.g. in the case of **24** and **25**, the heterocyclic nitrogen potency is increased by a push-pull effect [226].

In addition to its nitrogen HBA group, the heterocycle can interact with P-gp by aromatic hydrophobic interaction. The lack of activity of pyridine and quinoline derivatives **2-5** seems to indicate that hydrophobic interactions are rather involved. Differences in the observed activities of the compounds can be attributed to the spatial position of the amino HBA

groups and aromatic rings. The results display that two long chains allow the best interactions between compound pharmacophoric groups and P-gp binding sites (Figure 11.).

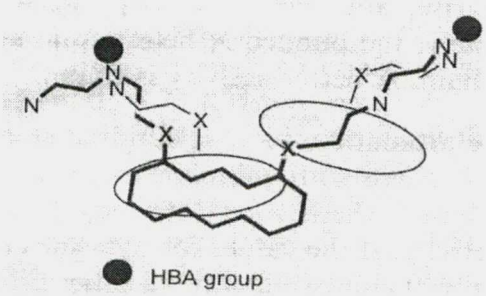


Figure 11. A possible superimposition of pyridoquinoline derivatives (in bold) and acridine derivatives when interacting with the P-gp

6.2.2. Reversal of multidrug resistance in mouse lymphoma cells by benzo [b]-1,8-naphthyridine derivatives

Tricyclic derivatives including phenotiazines and acridines are among compounds showing anti MDR activity [214, 215]. Unfortunately, most of these agents suffer clinically from their intrinsic toxicity or from side-effects at concentrations which neutralise P-gp [227]. So to design new inhibitors active at low concentrations and free of serious side-effects is vital. Benzonaphthyridine nucleus was selected because of its structural similarity with acridines, the more so as compounds in this chemical series are usually less toxic than acridines..

According to the data received there is clear evidence that benzonaphthyridine derivatives increased accumulation of Rhodamine-123 in MDR cells more efficiently than phenotiazines and acridine derivatives, or the positive control verapamil did. Since the hydrophobic moiety could be essential in the activity of MDR modulators, benzonaphthyridine derivatives with the polycyclic nucleus modified in various ways were tested. The activity of benzonaphthyridine derivatives with various side-chain was compared with the aim of determining the effect of the nature of this chain on the MDR reversion. Compounds **5**, **6**, **8** and **10**, which bore saturated aza heterocyclic moieties (pyrrolidine, morpholine, piperidine), were the most active at the lowest concentration. In addition, the presence of the amino group protonatable in the physiological conditions seemed to be necessary for the reversion of MDR in the case of tricyclic derivatives. This is clearly demonstrated by comparing **3** to **11**. Indeed, there is the same lipophilic moiety in both compounds but there is an acetamido group in the side-chain of **11** whilst the amino group is protonatable in **3**. Similar results were obtained by Hever et al for acridine derivatives [215].

7. CONCLUSION

1. Among trifluoro-methylketones, 4,4,4-trifluoro-1-phenyl-1,3-butanedione (TF10), 1,1,1-trifluoro-3-(4,5-dimethyloxazol-2-yl)-2-propanone (TF11) and 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) has the strongest antibacterial effect on *E. coli* AG100 wild and on *E.coli* AG100A proton pump deleted mutant strain as well.

2.a.) 4,4,4-trifluoro-1-phenyl-1,3-butanedione (TF10) and 1,1,1-trifluoro-3-(4,5-dimethyloxazol-2-yl)-2-propanone (TF11) showed moderate combined effect on both *E. coli* strain in combination with Ca-calmodulin antagonistic promethazine. 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) exerted synergy with promethazine only on the wild *E. coli* strain indicating that this phenotiazine can be a target of the AcrAB efflux pump.

b.) The most effective trifluoro-methylketone, 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) was studied in interaction with Ca-channel blocker verapamil. On mutant strain the combination was additive, however on the wild strain an antagonism was found. It is suggest that TF 18 probably makes differences between the calmodulin-antagonistic and Ca-channel blocker property, and the proton pump has some role on these effects.

3. Promethazine eliminated the pBR322 plasmid at a maximal rate from mutant strain at lower concentration than from the wild type indicating that the presence of this efflux pump can influence the plasmid elimination with promethazine.

4. 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) decreased 32 fold the MIC value of erythromycin in wild, and 4 fold in mutant strains respectively. The sensitivity of ampicillin did not change significantly in combination with TF 18 in both strain. It indicates that TF 18 does have a special effect on inhibiting this efflux pump. The weaker synergism of TF18 and ER in mutant strain can be explained by the strong antibacterial effect of TF18.

5. Different proton pump inhibitors were compared in combination with erythromycin on both strains. Neither Omeprasol and nor 1-(2-benzoxazolyl)-2-propanone (TF51) decreased the sensitivity of erythromycin on both strain indicating that there is a certain structural requirements for the inhibition of AcrAB efflux pump.

6. Known resistance modifiers were combined with antibiotics on both *E. coli* strains.

a.) Erythromycin with promethazine was additive on wild and mutant strain as well. Erythromycin with verapamil was indifferent on wild strain, however on mutant strain this combination was synergystic. The latter phenomenon is explained by a changing effect of verapamil in the membrane permeability, which cannot be specific for its Ca-antagonistic property, since the Ca-calmodulin antagonistic promethazine did not show similar effect.

b.) The ampicillin with both Ca-antagonists stood in antagonism in mutant strain, namely the Ca-channel blockers somehow could decrease the membrane permeability in this strain. The effect of verapamil on mutant strain depends on the property of antibiotics combined with VP: with hydrophobic erythromycin synergism, with hydrophilic ampicillin antagonism were found, indicating the difference membrane permeability requirements of the two antibiotics.

7. Among trifluoro-methylketones the motility of *P. vulgaris* was inhibited by the strongest way by -(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) reinforcing its proton pump inhibitory action supposed in the interaction studies. Furthermore a progressivity is phenomenal for the antimotility effect of TF**18**: first the running cells become tumbling and with increasing of the drug concentration, the tumbling ones become non-motile.

8. For the strong antibacterial, antimotility and so for the proton pump inhibitory effect of the trifluoro-methylketones, the structural requirements are the follows: 1.presence of the benzoxazol ring, since benzazole derivatives were more active than benzene or azole ones next to intact side chain. Next to the presence of benzoxazol ring 2. presence of side chain found in TF 18 is essential, since removal of terminal F_3 group, methylene group resulted drastic decreasing the antibacterial and antimotility action as well.

9. The MDR reversal activity of acridine derivatives depends on the aromatic moiety, the bounded heteroatom and nature of the side chain. Better activity is found: when the aromatic moiety is acridine or pyridoquinoline compared with quinoline and pyridine. Keeping the same atom on the ring, derivatives, having two side long chains, are more effective than ones, having one side short chain. Anti MDR activity was correlated with the presence of two or three hydrogen bond acceptor (HBA) groups. Possible HBA groups are tertiary amino groups, heterocyclic nitrogen like in the case of 2,8,10-Trimethyl-bis-4,6-[N-(2'-diethylaminoethyl)-N-methylamino] pyrido-[3,2-g] quinoline (24) and 2,8,10-Trimethyl-6-phenoxy-4-[N-(2'-diethylaminoethyl)-N-methylamino] pyrido-[3,2-g] quinoline (25). In addition to its nitrogen HBA group, the heterocycle can interact with P-gp by aromatic hydrophobic interaction.

10. The activity of benzonaphthyridine derivatives with various side-chain was compared with the aim of determining the effect of the nature of this chain on the MDR reversion. Compounds, which bore saturated aza heterocyclic moieties (pyrrolidine, morpholine, piperidine) were the most active. The presence of the amino group protonatable in the physiological conditions seemed to be necessary for the reversion of MDR.

8. SUMMERY

With the more and more emerging multidrug resistance problem, there is urgent need to research the cause, nature, and spread of the resistance, and to find some solutions for reversing MDR both in bacteria and tumour cells. One of the solutions is to develop new and more effective antibiotics and citostatics, but in a few time the appropriate resistance mechanism will arise to make the drugs ineffective. An other way for preventing the appearance of multidrug resistance is the application of combined chemotherapy, particularly in treatments of long-time duration e.g. certain type of cancers and tuberculosis. However it is better, when the resistance mechanisms are inhibited by some chemical agents. During the treatments of bacterial infections there already have been a method working with reversing of resistance, where the β-lactam antibiotics are combined with β-lactamase-inhibitors e.g. amoxicillin + clavulanic acid, ampicillin + sulbactam or piperacillin + tazobactam. Theoretically the elimination of plasmids carrying the resistance genes could also mean a solutions, however the elimination never occurs in the whole bacterial population. A better solution is to inhibit such a resistance mechanism, where the inhibition exists among all the cells of the population. The inhibition of bacterial and tumour multidrug efflux pumps by some chemical agent can be a practicable plan to overcome MDR. Among Gram-negative bacteria the intrinsic resistance for some typically „Gram-positive antibiotics" e.g. erythromycin and for some unrelated drugs, is on the one hand due to the working of an efflux pump system which uses proton gradient for its operation. The successful inhibition of this proton pump by 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF 18) means the possibility to employ an earlier ineffective antibiotics with a pump inhibitor in combination. The experiments show, that a so-called „Gram-negative resistance" can be overcome by this method. The way of the reversal mechanism, namely the inhibition of proton pump by TF 18, was proved by the successful inhibition of bacterial movement.
The reversal of drug resistance by inhibition of P-glycoprotein 170 efflux pump in mouse lymphoma cells was successful with chemically unrelated agents as well. The relationship found between the biological activity and chemical structures provides information for the further drug design research. The results are derived from model experiments. All the new information received can serve as a building stone in the research field of multidrug resistance reversary. First of all the statements of structure-activity relationship may be important for further drug design, which involves the synthesis of more effective and less toxic compounds and for *in vivo* experiments in the future.

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ACKNOWLEDGEMENTS

First of all I would like to express my deepest and sincere gratitude to my supervisor ***Professor József Molnár*** for introducing me to the scientific research. From both professional and human aspects, it was a great pleasure to work under his guidance and to learn so much about microbiology and oncology. His wild knowledge, understanding, encouraging and valuable advices served as an excellent basis for the preparation of this thesis.

I would like to express my sincere gratitude to ***Prof. Yvette Mándi***, chair of the department, for providing possibility to work at the department.

I express my special thanks to ***dr. Csilla Miskolci***, ***dr. Diána Szabó*** who helped me very much as colleagues and as friends during my first years at the department. Many thanks to ***Gabriella Spengler***, ***Annamária Molnár*** and ***Mrs Anikó Vigyikán*** and all my other colleagues from the Department of Medical Microbiology and Immunobiology, who all supported me and created a pleasant working environment.

I am grateful to ***Dr. Andreas Varga*** (Humboldt University, Berlin, Germany), ***Prof. Derek Sharples*** (University of Manchester, United Kingdom) and ***Prof. Theresia Thalhammer*** (University of Vienna, Austria) who gave me possibility to work for short scientific mission at their laboratories.

I owe ***Dr. Imre Ocsovszki*** my thanks for performing the flow-cytometric measurements.

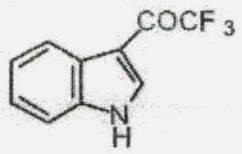
This study has been supported by various organization including "Foundation for Cancer Research Szeged", COST Action 916 Grant and TÉT/BILAT D-99/96 German-Hungarian Grant.

I owe a dept of gratitude to my beloved parents who taught me persistence and always supported, encouraged me and taught me optimistic thinking. Many thanks to my sisters for their understanding and support. I would like to express my special thanks to my husband for his understanding, support and patience.

I dedicate this thesis to the memory of my mother.

ANNEX

1. Chemical structures of trifluoro-methylketones



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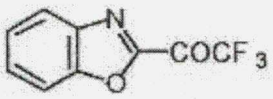

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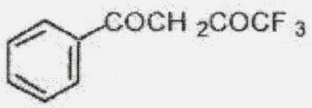

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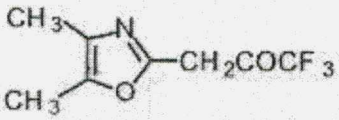

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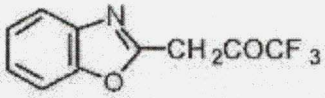

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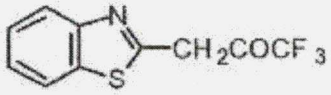

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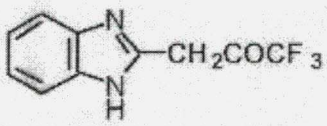

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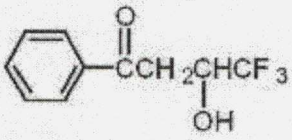

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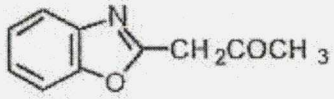


TF51

5: *3-trifluoroacetylindole* (obtained from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan),

6: *2-trifluoroacetylbenzoxazole* [175],

10: *4,4,4-trifluoro-1-phenyl-1,3-butanedione* [176.],

11: *1,1,1-trifluoro-3-(4,5-dimethyloxazol-2-yl)-2-propanone* [176],

18: *1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone* [176],

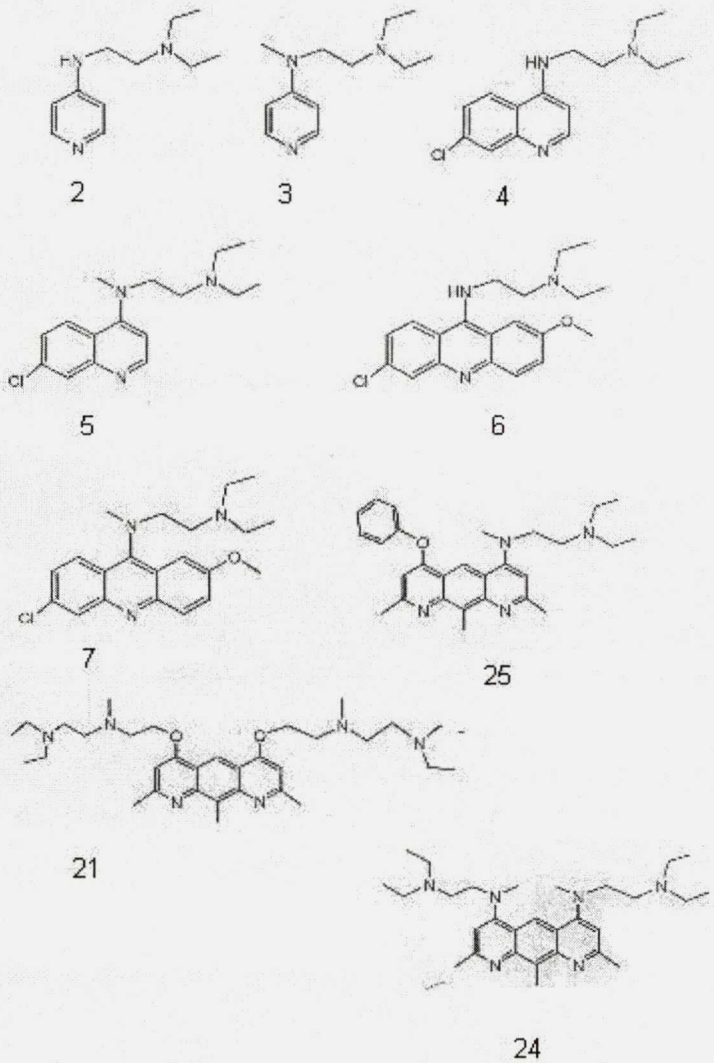
19: *3-(2-benzothiazolyl)-1,1,1-trifluoro-2-propanone* [177],

20: *3-(2-benzimidazolyl)-1,1,1-trifluoro-2-propanone* [177],

50: *3,3,3-trifluoro-2-hydroxypropyl phenyl ketone* [175],

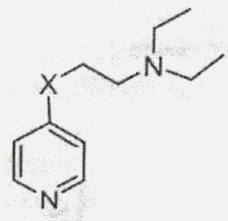
51: *1-(2-benzoxazolyl)-2-propanone*

2. Chemical structures of acridine derivatives: aza mono, bi and tricyclic compounds

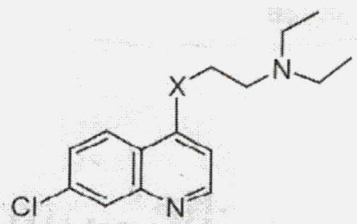


2: *4-[(2'-Diethylaminoethyl)amino]-pyridine*
3: *4-[(2'-Diethylaminoethyl) methylamino]-pyridine*
4: *7-Chloro-4-[(2'-diethylaminoethyl)amino]-quinolone*
5: *7-Chloro-4-[(2'diethylaminoethyl)-methylamino]-quinolone*
6: *6-Chloro-2-methoxy-9-[(2'-diethylaminoethyl)amino]-acridine*
7: *6-Chloro-2-methoxy-9-[(2'-diethylaminoethyl)methylamino]acridine*
21: *2,8,10-Trimethyl-4,6-bis[N-(2'-diethylaminoethyl)-N-methylamino) ethoxy] pyrido-[3,2-g] quinoline*
24: *2,8,10-Trimethyl-bis-4,6-[N-(2'-diethylaminoethyl)-N-methylamino] pyrido-[3,2-g] quinoline*
25: *2,8,10-Trimethyl-6-phenoxy-4-[N-(2'-diethylaminoethyl)-N-methylamino] pyrido-[3,2-g] quinoline*

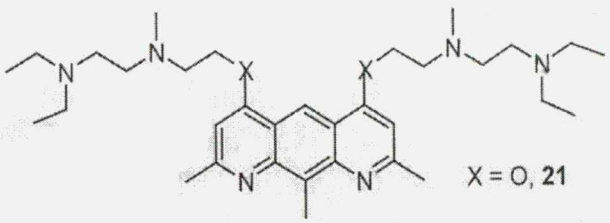
3. Chemical structures of acridine derivatives: aza mono, bi and tricyclic compounds with regard the X atom in the side chain



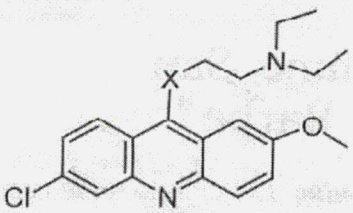
2 : X = NH
3 : X = NMe



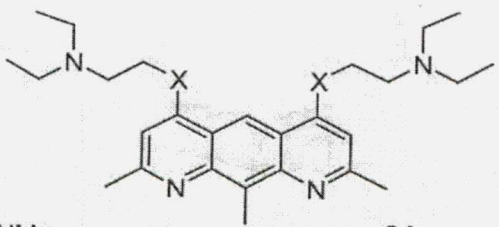
4 : X = NH
5 : X = NMe



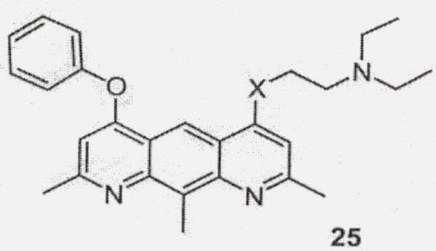
X = O, **21**



6 : X = NH
7 : X = NMe.

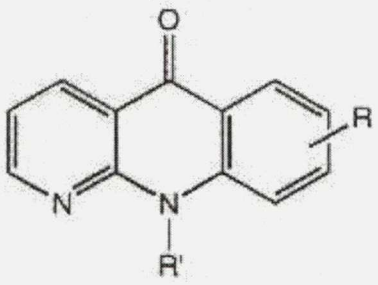


X = NMe **24**



25

4. Chemical structure of Benzo [b]-1,8- naphthyridine derivatives



Compounds	R	R'	Type of nucleus
1	-	10-(2'-dimethylaminoethyl)	-5-one
2	-	10-(3'-dimethylaminopropyl)	-5-one
3	-	10-(2'-diethylaminoethyl)	-5-one
4	-	10-(2'-diisopropylaminoethyl)	-5-one
5	-	10-(2'-pyrrolidonoethyl)	-5-one
6	-	10-(2'-(N-methyl)-pyrrolidinoethyl)	-5-one
7	-	10-(2'-piperidinoethyl)	-5-one
8	-	10-(3'-piperidinopropyl)	-5-one
9	-	10-(3'-(N-methyl)-piperazinopropyl)	-5-one
10	-	10-(2'-morpholinoethyl)	-5-one
11	-	10-(2'-diethylacetamido)	-5-one

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